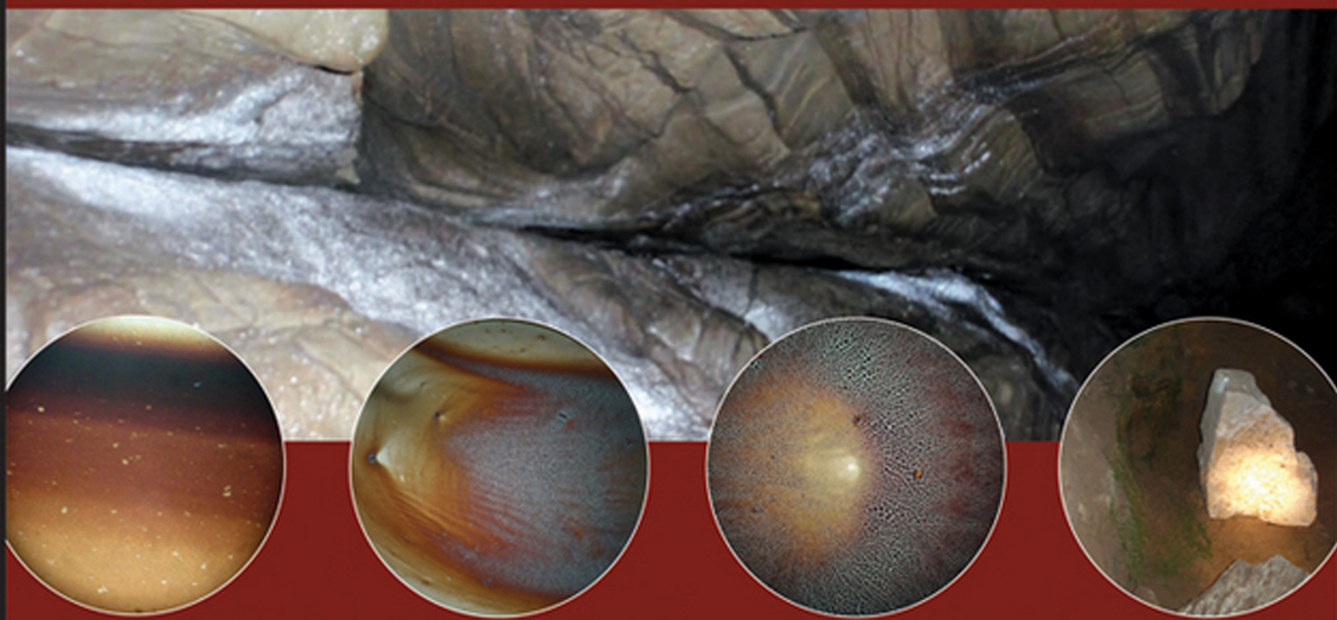


OM V. SINGH



EXTREMOPHILES

Sustainable Resources and
Biotechnological Implications

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EXTREMOPHILES



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Sustainable Resources and Biotechnological Implications

Edited by

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Published simultaneously in Canada.

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Library of Congress Cataloging-in-Publication Data:

Extremophiles : sustainable resources and biotechnological implications / edited by Om V. Singh.

p. cm.

Includes index.

ISBN 978-1-118-10300-5 (cloth)

1. Extreme environments—Microbiology. 2. Microbial biotechnology. I. Singh, Om V.

QR100.9.E987 2012

578.75'8—dc23

2012011093

Printed in the United States of America

10 9 8 7 6 5 4 3 2 1

*To Daisaku Ikeda, Uday V. Singh Chauhan, and Indu Bala,
in grateful appreciation of their encouragement*

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INTRODUCTION

Om V. Singh



It has long been in the interest of science to explore mysterious events to establish scientific theories. In the fascinating world of microorganisms, extremophiles are the most mysterious category of life on planet Earth (Rothschild and Mancinelli, 2001) and perhaps on other planets as well (Navarro-González et al., 2003, 2009). Nature, of course, offers abundant opportunities to life forms that can consume or produce sufficient energy for their survival. However, normal survival may not be possible in environments that experience extreme conditions (e.g., temperature, pressure, pH, salinity, geological scale and barriers, radiation, chemical extremes, lack of nutrition, osmotic barriers, or polyextremity). Due to extraordinary properties, certain organisms (mostly bacteria and archaea, and a few eukaryotes) can thrive in such extreme habitats; they are called *extremophiles*.

It would benefit human society to learn from extremophiles; they have the potential to assist us in dealing with emerging diseases, due to their ingenious adaptations and the metabolic strategies they use to survive under extreme environmental conditions. The products of extremophilic microbial metabolisms are referred to as *extremolytes*: in the form of enzymes, proteins, and primary and secondary metabolic products, they have proven their importance to biotechnology. There has been some success in producing a variety of extremolytes on an industrial scale. Recent reports have covered various aspects of the current state of technologies involving metabolic products from extremophiles (Hammon et al., 2009; Brito-Echeverría et al., 2011; Burg et al., 2011). This book continues to bridge the technology gap and focus on aspects of extremolytes and the respective mechanisms regulating their biosynthesis that are relevant to human health, energy, and value-added products of commercial significance.

While attempting to learn from extremophiles, ignorance of extreme conditions is unjustifiable. Since the “deep time,” there have been extreme environments on Earth. With the wide-ranging ingredients of life in the atmosphere, it is inconceivable that life did not exist in geological time (i.e., 4.6 billion years ago). Little evidence of this time remains in Earth’s rocks; however, the existence of methanogens about 2.7 Gya (gigayears ago) has been proven by isotopic records, as stated by Chakravorty et al. in Chapter 1. The modern era allows for genetic adaptations, including horizontal and lateral gene transfer, among a variety of extremophiles, and the possibility of natural selection and/or spontaneous evolution remains. This chapter details the biochemical aspects and major events of molecular evolution, including the genomes and proteomes of various extremophiles, suggesting that modern technology can predict accurate evolutionary links among extremophiles.

In extreme environmental niches, uncultivable microorganisms can be found (Deppe et al., 2005). These microorganisms draw on unknown sources of energy, and modern

science has yet to discover a supporting growth medium that can be used with them. However, advancements in metagenomics may assist in exploration of the unique properties of such uncultivable microorganisms (B.K. Singh, 2010; Singh and MacDonald, 2010). If appropriate sources to grow uncultivable microorganisms can be found, it could open new doors to the fascinating microbial world and its unique characteristics. In Chapter 2, Chakravorty and Patra discuss the unique features of growth strategies for a wide variety of extremophiles, highlighting the methodologies and limitations.

The ocean covers 75% of the planet and is a diverse environment for life. Rasmussen (2000) presented evidence of deep-sea microfossils of threadlike microorganisms in 3,235-million-year-old volcanogenic sulfide deposits, representing the first fossil evidence for microbial life in a Precambrian submarine thermal spring system. Other studies have presented the facts of appropriate environment for all life forms due to the one significant element of life, water, which astrobiologists are exploring on other planets. After the discovery of hydrothermal vents in 1979, an entirely different ecosystem was observed there with a variety of prokaryotic and eukaryotic microorganisms that had adapted themselves to the hostile environment and the lack of energy from sunlight. The limited information and technology galvanized researchers to investigate microbial life under extremes of temperature, pressure, oxygen, pH, and so on. In Chapter 3, Aharon Oren presents facts and strategies for the isolation and cultivation of halophilic microorganisms. Arakawa et al. in Chapter 4 present unique properties of halophilic microorganisms and their manipulation toward aimed biotechnological applications. Then, in Chapter 5, Ximena C. Abrevaya presents the diverse features and applications of halophilic archaea.

Including the ocean, cold environments make up the majority of the biosphere on Earth and other planets. In Chapter 6, Garcia-Descalzo et al. present the facts that 90% of the ocean's volume is below 5°C and that sea ice (13% of the Earth's surface), glaciers (10% of the Earth's surface), and permafrost (24% of the Earth's surface) are full of living microorganisms. Other sites, such as lakes, deserts, caves, and the upper atmosphere (upper troposphere and lower stratosphere), are being considered as permanent cold environments for living organisms. The authors of this chapter also interpret the facts of molecular events and microbial modifications that allow them to survive in extremely cold environments.

Anoxia is another type of extreme condition in which microbes can live. Anoxic sites in the environment (i.e., deep underground, sedimented bottoms of water bodies, deep sea, higher altitudes, and industrial effluent sites) and gut microbial flora in animal systems reveal a vast variety of anaerobic bacteria that have long histories in chemical and fuel production (Zeikus, 1980). Francesco Cangelina in Chapter 7 discusses the ecological aspects of selective anaerobic extremophiles—thermophiles—and interprets the biotechnological implications of their thermal resistance.

Food is necessary for organisms to maintain the required energy levels for life. Regardless of the abundance of food on Earth, there are always concerns about food safety and security in human society. The advanced technologies of modern genetic engineering (GE) have potential to ensure food security, but food safety remains a topic of discussion (Singh et al., 2006). Food regulations imposed by government agencies (O.V. Singh, 2010) rely on data provided by food growers. The limited research efforts hamper our understanding of the impact of GE food on the living world. On the other hand, extremophiles, with their broad range of biotechnological implications, could prove suitable for food processing and production. Since ancient times, a variety of microorganisms have been used to produce fermented alcoholic beverages and other food products. Most organisms used in food

processing are mesophiles, but in some applications, extreme conditions are required. Microorganisms thriving in environments that are hostile to other organisms provide a source of novel bioproducts (extremozymes), products of primary and secondary metabolites. A broad category of these novel bioproducts is presented in Chapter 8 by Jane A. Irwin, who describes the unique roles of extremophiles and their bioproducts in food processing and production. This chapter adds to our understanding of whether extremophiles are able to fill the gaps in food safety that arise from GE food.

To meet the ever-increasing demand for energy, human society can rely on nature, which offers abundant renewable resources with the ability to replace fossil fuel. However, several issues, including economics and technological readiness, must still be resolved. Alternative fuel sources such as cellulosic ethanol or biodiesel are the most immediate and obvious target fuels. In Chapter 9, Taylor et al. discuss applications of extremophiles for biofuel research, and in Chapter 10, Chandel et al. examine how thermophiles are used in second-generation bioethanol production.

With the demand for ecofriendly bioproducts that can benefit biotechnology industries at the forefront, the exploration of microbial metabolic products has turned toward extremophiles. In Chapter 11, Agarwal and Mishra present ecofriendly applications of extremozymes in the textile industries. This chapter reveals that the use of extremozymes in everyday practical life may have additional applications that can fulfill biotechnology aims by reducing environmental pollution through toxic chemicals. In Chapter 12, Carlos A. Jerez discusses extremophilic applicability in the industrial recovery of metals.

Microbial metabolic products with unique characteristics, such as exopolysaccharides, represent a wide range of chemical structures with wide applications in the food, pharmaceutical, and other industrial fields. In Chapter 13, Barbara et al. present the fact that extremophiles are able to biosynthesize extracellular polymeric substances. These extremophiles could be another biofactory for exopolysaccharide biosynthesis. In continuation, Molina et al. in Chapter 14 present an overview of the biomedical applications of exopolysaccharides produced by microorganisms isolated from extreme environments.

Radiation in the form of particles or electromagnetic waves (i.e., ultraviolet radiation, gamma rays, x-rays, radio waves, etc.) causes serious oxidative damage to vital biomolecules, including proteins and nucleic acids. Historically, ultraviolet radiation and other radioactive substances have been linked to many harmful effects, including immune suppression, dermatitis, premature aging, neurodegeneration, and skin cancer. Extremolytes are unique organic compounds that are not directly involved in the normal growth, development, or reproduction of organisms; however, their absence does affect the long-term impairment of the organism's survivability, fecundity, or aesthetics. These microbial reserves have been widely explored for industrial significance; however, their therapeutic implications remain to be investigated. The exploration of strategic therapeutic applications of extremophiles in the area of defense and homeland security has credible potential. The potential for development of radioprotective drugs using radioresistant extremophiles has yet to be determined. In Chapter 15, Copeland et al. discuss the biosynthesis of extremolytes along with the concept of therapeutics utilizing the unique properties of radiation-resistant microorganisms. In Chapter 16, Kumar and Singh present smart therapeutics that can be produced from extremophiles. A brief description of the unexplored applications will provide industrial professionals with an opportunity to think outside the box by making investments in research and technology development.

Driven by increasing industrial demands for biocatalysts, enzymes, and metabolites that can cope with industrial process conditions, considerable effort has been made to search for such products. Because of their ability to thrive in extreme habitats that would kill other organisms almost instantly, extremophiles have a strong potential for future advancements in biotechnology, pharmaceuticals, and the extermination of certain toxic compounds from the environment. Extremozymes, such as thermostable amylase, are being incorporated into biochemical reactions that occur at high temperatures in water-based solutions, and could be substituted for high-cost reactants to lower the cost of the final product. Furthermore, the current nuclear arms race, instability in the environment due to ozone depletion, and solar flares reaching to the Earth's surface (M8.7 Solar flare and Earth Directed CME available at http://www.nasa.gov/mission_pages/sunearth/news/News012312-M8.7.html) make normal life vulnerable to natural and human-made radiation. Radiation-resistant microbes contain compounds that can potentially be harnessed as radioprotective drugs, which may be useful in space programs to prevent unwanted radiation exposure. In the years to come, the exploitation of extremophiles will indubitably advance to find the cures for diseases such as radiation-mediated cancer and meet other industrial demands.

This book is a collection of outstanding articles elucidating several broad-ranging areas of progress and challenges in the utilization of extremophiles as sustainable resources in the biomedical and biotechnological fields. The book will contribute to research efforts in the scientific community and commercially significant work for corporate businesses. The expectations are to establish long-term sustainable alternatives for adverse environmental conditions from microorganisms living under extreme conditions. Apart from therapeutics, this book also emphasizes the use of sustainable resources (i.e., extremolytes and extremozymes) for value-added products, which may help in revitalizing the biotechnology industry on a broader scale.

We believe that readers will find these articles interesting and informative for their research pursuits. It has been my pleasure to put this book together with Wiley-Blackwell. I would like to thank all of the contributing authors for sharing their outstanding research and ideas with the scientific community.

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MOLECULAR EVOLUTION OF EXTREMOPHILES

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1.1. INTRODUCTION

Extremophiles have evolved to adapt to severe geological conditions. Their adaptation cannot be justified merely as stress responses, as they not only survive but thrive in such milieus. The term *extreme* is used anthropocentrically to designate optimal growth for extremophiles under such conditions. This can be proved through the time-evolved complexity pertaining to their molecular mechanism of adaptation. Their evolution can be mapped on the geological time scale of life, in which a thermophile is argued to be the last common ancestor from which life has arisen. The early existence of methanogens has been proved

Extremophiles: Sustainable Resources and Biotechnological Implications, First Edition. Edited by Om V. Singh.
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by isotopic records about 2.7 Gya (gigayears ago). Extremophiles span the three domains of life and not only thrive on Earth but also occupy extraterrestrial space. One of the most impressive eukaryotic polyextremophiles is the tardigrade, a microscopic invertebrate found in all Earth habitats (Romano, 2003). Extremophiles such as archaea have evolved through the phenomenon of lateral gene transfer by orthologous replacement or incorporation of paralogous genes (Allers and Mevarech, 2005). It has been suggested that the switch from an anaerobic to an aerobic lifestyle by the methanogenic ancestor of haloarchaea was facilitated by the phenomenon of lateral gene transfer of respiratory chain genes from bacteria. They also possess coregulated genes in operons, leading to coinheritance by lateral gene transfer (Allers and Mevarech, 2005).

According to the 16S rRNA classification domain, archaea can be divided into four kingdoms: Crenarchaeota, Euryarchaeota, Korarchaeota, and the recently discovered Nanoarchaeota (Grant and Larsen, 1989; Huber et al., 2002). Crenarchaeota includes thermophiles and psychrophiles, Euryarchaeota comprises hyperthermophiles and halophiles, hyperthermophilic archaea represent Korarchaeota, and to date, Nanoarchaeota is represented by only a single species, *Nanoarchaeum equitans*.

Recently, sequencing of extremophilic genomes and their global analysis has shed light on their genetic evolution. An appropriate example of such an evolutionary process is that of the radioactive damage-resistant microorganism *Deinococcus radiodurans*. *Deinococcus* possesses a robust DNA repair system that performs interchromosomal DNA recombination to cope up with radiation damage of its genome. The natural selection pressure that led to this novel evolution is known as *desiccation* (Cavicchioli, 2002). These organisms educate us on exactly what extremophilic evolution means.

The evolutionary history of extremophiles will aid us in understanding the adaptation of microbes to extreme conditions and environments. Research on extremophiles speeded up only recently after the first genome sequence of the methanogenic archaeon *Methanococcus jannaschii* was published (Bult et al., 273). Success in extremophilic research is limited, due to the lack of proper in vitro conditions and genetic systems for extremophile cloning, library creation, and expression (Allers and Mevarech, 2005). Knowing the evolutionary route of extremophiles can lead to novel discoveries, adding to industrial economy. In-depth understanding of extremophilic evolution will help in engineering extremophiles and hence add to the multibillion-dollar biotechnology industry. In this chapter we discuss the natural routes followed by extremophiles in their evolution and in ways that they can be engineered in vitro.

1.2. MOLECULAR EVOLUTION OF THERMOPHILES

1.2.1. Habitat

Thermophilic environments are widespread throughout the Earth, encompassing hot springs, volcanic areas, geothermal vents and mud holes, and solfataric fields (Huber et al., 2000). They are found in several parts of the world, the largest being Yellowstone National Park in the United States. These ecosystems have high salt concentrations (3%) and slightly acidic-to-alkaline pH values (pH 5 to 8.5) (Bock, 1996). In volcanic regions large amounts of steam are formed which contain such gases as carbon dioxide, hydrogen sulfide, methane, nitrogen, carbon monoxide, and traces of ammonia or nitrate. Coal refuse piles and hot outflows from geothermal power plants constitute artificial high-temperature milieus (Huber et al., 2000). Domestic and industrial hot-water systems are the anthropogenic habitats for thermophiles.

1.2.2. Cellular Organization

In the cell membranes of thermophiles, the lipid side-chain branching and ether linkages of the phospholipid bilayer contribute to their thermoadaptation (van de Vossenberg et al., 1995; Mathai et al., 2001; Futterer et al., 2004). A few extreme thermophiles such as *Pyrobolus* and *Thermoplasma acidophilum* use a modified lipid that forms a monolayer instead of a bilayer, thus making it immune to the tendency of high temperature to pull bilayers apart. In thermophiles growing above 60°C, modifications are also observed in the metabolic pathways. Synthesis of heme, acetyl-CoA, acyl-CoA, and folic acid are either reduced or absent in thermophiles (Kawashima et al., 2000).

1.2.3. Genome

Thermophiles have adapted to temperature maxima by the evolution of a wealth of structural and functional features. Recently, genomes of hyperthermophilic archaea, *Nanoarchaeum equitans* and *Thermococcus kodakaraensis*, *Sulfolobus acidocaldarius*, and *Carboxydotherrmus hydrogenoformans* were sequenced, providing insight into high-temperature evolution of their genome and metabolic versatility in specific thermophilic environments (Podar and Reysenbach, 2006). It has been observed that the primary structure of DNA is prone to denaturation at increased temperature by hydrolysis of the *N*-glycosyl bond and due to deamination of the cytosine bases (Kampmann, 2004). The genomes of such thermophiles as *T. kodakaraensis* show a high guanine–cytosine (GC) content (Bao et al., 2002; Saunders et al., 2003). They also show a preference for purine-rich codons, favoring charged amino acids (Paz et al., 2004). DNA shows positive supercoils in thermophiles, hence greater stability. The positive supercoils are catalyzed by the enzyme reverse gyrase, which is present only in hyperthermophiles, in contrast to the negative supercoils in mesophiles (Madigan, 2000). Additionally, monovalent and divalent salts enhance the stability of nucleic acids by screening the negative charges of the phosphate groups, protecting DNA from depurination and hydrolysis (Rothschild and Mancinelli, 2001). In some hyperthermophiles, heat-resistant protein (histone-like protein) binds and stabilizes the DNA by lowering its melting point (T_m) (Madigan, 2000). Recently, work carried out by Zeldovich et al. (2007) concludes that an increase in purine (A + G) of thermophilic bacterial genomes due to the preference for isoleucine, valine, tyrosine, tryptophan, arginine, glutamine, and leucine, which have purine-rich codon patterns, is responsible for the possible primary adaptation mechanism for thermophilicity. These amino acid residues increase the content of hydrophobic and charged amino acids, enhancing thermostability.

1.2.4. Proteome

Thermophiles are under constant threat of high temperature on their proteins. Thus, thermophilic intracellular protein and enzymes, compatible solutes, molecular chaperones, and translational modifications lead synergistically to their dramatic stability at high temperature (England et al., 2003). Hyperthermophilic proteins are more resistant to denaturation due to restriction on the flexibility of these proteins (Scandurra et al., 1998). Factors such as increased van der Waals interactions, higher core hydrophobicity, hydrogen bonds, ionic interactions, coordination with metal ions, and compactness of proteins contribute to thermostability (Berezovsky et al., 2007). The presence of increased salt bridges in proteins of

thermophiles has been a unanimous observation claimed by many researchers (Scandurra et al., 1998). In thermophiles we also observe proline in β -turns, giving rise to rigidity in proteins. Change in amino acids from Lys to Arg, Ser to Ala, Gly to Ala, Ser to Thr, and Val to Ile have been observed among mesophilic to thermophilic organisms (Scandurra et al., 1998). Pertaining to secondary structure, thermostable proteins have high levels of α -helical and β -sheet content. They also have a slow unfolding rate, which helps to retain their near-native structures. For example, pyrrolidone carboxyl peptidase from *Pyrococcus furiosus* and ribonuclease HII from the archaeon *Thermococcus kodakaraensis* show slower unfolding rates than those of their mesostable homologs, which vary from one mesophile to another (Okada et al., 2010). An enhanced hydrophobic effect is one of the reasons for the phenomenon of slow unfolding of thermophilic proteins (Okada et al., 2010). Integral membrane proteins of thermophiles avoid glutamine, lysine, and aspartate amino acid residues, unlike soluble proteins, as a mode of adaptation to increased temperature (Lobry and Chessel, 2003). Larger amounts of Ala, Gly, Ser, Asp, and Glu and smaller amounts of Cys have been reported in the transmembrane proteins of thermophiles (Lobry and Chessel, 2003). A high GC content in the genome leads to the coding of GC-rich codons for amino acids such as Ala, Pro, Trp, Met, Gly, Glu, Arg, and Val. Also synthesized are chaperonins, which refold denatured protein: for example, the thermosome of hyperthermophiles capable of growth above 100°C, such as *Pyrolobus fumarii* and *Methanopyrus kandleri* (Madigan, 2000).

1.3. MOLECULAR EVOLUTION OF PSYCHROPHILES

1.3.1. Habitat

Cold environments constitute the largest biome on Earth. Almost 70% of Earth's surface is made up of oceans that have a temperature of 4 to 5°C, and 15% are polar regions. In such milieus the evolutionary pressures are high salt concentrations in oceans, ultraviolet (UV) radiation on the surface of glaciers and ice, and low water and nutrient concentrations in endolithic rocks of antarctic dry deserts (Feller and Charles, 2003).

1.3.2. Cellular Organization

Psychrophiles have an evolved lipid bilayer to avoid gel-phase transition and drastic loss of membrane properties (Feller and Charles, 2003). This is achieved through reduction in the packing of acyl chains in the membrane and introduction of steric hindrance, which reduces membrane viscosity (Feller and Charles, 2003). Psychrophiles have a larger proportion of polyunsaturated and branched fatty acids, to increase membrane fluidity. *Cis*- and *trans*-unsaturated double bonds are observed in the acyl chain, which induce a bend and a kink, respectively, resulting in lowered compactness in the lipid bilayer. Shorter fatty acyl chains, polar pigments, and membrane-bound carotenoids also increase membrane flexibility. The presence of cryoprotectants enhances their nutrient uptake. Oxygen is more soluble at low temperatures; hence, cells are prone to oxidative damage. In response to this, metabolic reactions that produce reactive oxygen intermediates are eliminated. Elimination of molybdopterin-dependent metabolism in *Pseudoalteromonas haplokantis* is an example of this (Podar and Reysenbach, 2006). Psychrophiles also possess a large number of gas

vesicles, which leads to reduction in the cytoplasmic volume, resulting in shorter diffusion times (Staley et al., 1989). Trehalose and exopolysaccharides (EPSs) in psychrophiles play a role in cryoprotection by preventing protein denaturation and aggregation (Phadtare, 2004). EPSs are highly hydrated molecules secreted by psychrophiles in the antarctic marine environment that assist their survival strategy by providing a protective envelope to the cells and the extracellular proteins (Nichols et al., 2005).

1.3.3. Genome

Psychrophiles produce nucleic acid-binding proteins such as RNA helicase to relieve strong interactions between DNA strands and secondary structures in RNA which impair transcription, translation, and replication (Feller and Charles, 2003). For example, an antarctic archeon synthesizes an RNA helicase, which removes cold destabilized RNA secondary structures. To reduce oxidative damage the genome possesses a greater number of catalase and superoxide dismutase genes (Podar and Reysenbach, 2006). In psychrophilic bacteria, incorporation of dihydrouridine in tRNA is observed, which adds to the conformational flexibility of RNA (Feller and Charles, 2003).

1.3.4. Proteome

Psychrophiles are adapted to survive below-freezing temperatures. Like thermophiles, they have evolved parallel strategies for their survival. A handsome strategy employed is that they translate cold-adapted enzymes. The adaptations can be achieved through numerous molecular mechanisms. Structural factors include reduction in electrostatic interaction and increase in hydrogen bonding (D'Amico et al., 2006). In 2003, Feller and Charles reported that nucleic acid-binding proteins play key roles in protecting psychrophilic genomes and identified genes that encode such proteins. Like molecular chaperones, cold-shock proteins assist in protein renaturation during the growth of psychrophiles (Berger et al., 1996). They also increase translation efficiency by destabilizing secondary structures in mRNA (Podar and Reysenbach, 2006). The presence of unique antifreeze proteins such as the proteins from *Marinomonas primoryensis* and *Pseudomonas putida* GR12-2 lowers the freezing point of cellular water (Feller and Charles, 2003). Moreover, cold-adapted enzymes possessing a flexible catalytic center show high specific activity at low temperatures (Podar and Reysenbach, 2006). Survival of psychrophiles in temperatures at the other end of the thermometer is brought about by cold acclimation proteins (Feller and Charles, 2003). An example is the RNA chaperone CspA. The presence of antifreeze peptides and glycopeptides in psychrophilic eukaryotes lowers the freezing point of cellular water by binding to ice crystals during their formation (Feller and Charles, 2003). An increase in the flexibility of proteins is the key to low-temperature adaptation. This is achieved by decreasing the number of Pro and Arg residues (which leads to the rigidity of proteins by restricting backbone rotations) and increasing the number of Gly residues in their sequences (Feller and Charles, 2003). The protein interiors are less hydrophobic, decreasing their compactness, and weak noncovalent interactions are minimized. The protein surface shows the exposure of nonpolar groups and acidic residues which interact strongly with the essential water layer required to maintain the integrity of the protein structures.

1.4. MOLECULAR EVOLUTION OF HALOPHILES

1.4.1. Habitat

Hypersaline environments are widely distributed on Earth. They are of various forms, including natural permanent saline lakes and salt marshes. They have also been created artificially, due primarily to anthropogenic activities and exist as solar salterns (Setati, 2010). Hypersaline environments can be classified as thalassohaline and athalassohaline. The former were created by the evaporation of seawater. Sodium and chloride ions dominate and the pH is nearly neutral. Athalassohaline environments have a higher concentration of divalent cations than the monovalent ions and a low pH (Oren, 2002).

1.4.2. Cellular Organization

Halophiles need to survive in environments of high salt concentration; hence, they maintain cellular osmotic pressure by controlling the amount of salt inside a cell. First, they possess Na^+/H^+ antiporters to maintain a low sodium ion concentration (Oren, 1999; Zou et al., 2008). The Halobacteriales, including fermentative or homoacetogenic anaerobes, accumulate K^+ and Na^+ ions to maintain osmotic balance. Second, enhanced levels of glycerol, amino acids, alcohols, and their derivatives (e.g., glycine, betaine, and ectoine) in their cells maintain their osmolality (Galinski, 1995; Shivanand and Mugeraya, 2011).

The lipid bilayer membranes of halophiles contain phosphatidyl glycerol and phosphatidyl glycerol sulfate (PGS). Glycolipids and PGS are the taxonomic markers of halophilic archaea (Kamekura, 1993; Upasani et al., 1994). An interesting feature of halobacterial cells is that they lack intracellular turgor pressure, leading to the formation of corners in their cells (Schleifer and Stackebrandt, 1982).

1.4.3. Genome

The GC content of the halophilic genome is around 60 to 70% (Siddiqui and Thomas, 2008). High GC levels can avoid ultraviolet-induced thymidine dimer formation and mutations. Adaptations to a hypersaline environment are achieved through lateral gene transfer. At the DNA level, compared to nonhalophilic genomes the halophiles exhibit distinct dinucleotides (CG, GA/TC, and AC/GT) at the first and second codon positions, reflecting an abundance of aspartate, glutamine, threonine, and valine residues in halophile proteins, which leads to their stability (Paul et al., 2008). The presence of high levels of CG dinucleotides leads to an increase in stacking energy and, thus, genome stability.

1.4.4. Proteome

The halophilic proteins are magnificiently engineered naturally to possess less hydrophobicity, as at high salt concentrations, proteins are destabilized by high hydrophobic interactions, which leads to protein aggregation. One remarkable feature of halophilic proteins is that they are often also thermotolerant and alkaliphilic (Setati, 2010). Acidic amino acid residues dominate their surface because they can hold the essential hydration shell layer for stability and catalysis intact at the surface of the protein. This is reflected in the low pI values of halophilic proteins (Siddiqui and Thomas, 2008). Acidic residues also form salt bridges, which add to the rigidity and thus stability of protein structures. A decrease

in large hydrophobic residues and an increase in smaller residues also contribute to the stability of halophilic proteins. Moreover, secreted enzymes are attached by lipid anchors in the halophilic bacterium *Salinibacter ruber* (Podar and Reysenbach, 2006). In relation to the secondary structure of proteins it was reported that residues that have low propensities for forming α -helices are preferred more in halophiles than in nonhalophiles. This leads to an increase in protein flexibility at high salt concentrations (Paul et al., 2008).

1.5. MOLECULAR EVOLUTION OF ALKALIPHILES

1.5.1. Habitat

Alkaliphiles live in soils rich in carbonate and in soda lakes and can be classified into alkaliphiles and haloalkaliphiles. Along with their extreme alkaline habitats, alkaliphiles also coexist where neutrophilic microorganisms dwell. They also thrive in deep-sea sediments and hydrothermal areas (Horikoshi, 1998). Naturally occurring alkaline environments are soda deserts and soda lakes. The alkaline lakes and desserts are geographically widely distributed. The Wadi Natrun lakes in Egypt are the best example of alkaline milieus. Alkaline environments have formed through the leaching of metal bicarbonates from rocks. Saline brines are also rich in divalent cations. Saturation of groundwater with respect to CaCO_3 results in the deposition of calcite and leads to their alkalinity (Seckbach, 2000). Small natural environments such as the gut of termites and artificial environments such as fermented foods also harbor alkaliphiles (Horikoshi, 2010). Artificial alkaline environments are created by permanent alkaline effluents through anthropogenic activities.

1.5.2. Cellular Organization

Alkaliphiles maintain nearly neutral pH by constantly pumping protons into their cytoplasm (Horikoshi, 1999). For example, *Bacillus subtilis* and *Vibrio alginolyticus* have evolved a mechanism for acidification of cytoplasm relative to the external pH (Speelmans et al., 1993). The cell membrane is constructed of acidic polymers such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, teichuronic acid, and phosphoric acid, which reduce the pH at the cell surface (Aono and Horikoshi, 1983). The acidic polymers permit absorption of sodium and hydronium ions and repel hydroxide ions, permitting the cell to grow at alkaline pH. Passive regulation of cytoplasmic pools of polyamines, low membrane permeability (Bordenstein, 2008), and Na^+/K^+ antiporters (Hamamoto et al., 1994) maintain pH homeostasis in alkaliphiles.

1.5.3. Genome

Alkaliphiles have evolved genetically to cope with their environment. Recently, the complete genome of *Bacillus subtilis* and *Bacillus halodurans* C-125 has been sequenced. Genes responsible for the alkaliphily of *B. halodurans* C-125 and *Bacillus firmus* OF4 have been analyzed (Takami et al., 1999). In their genome, several open reading frames for Na^+/H^+ antiporters responsible for pH homeostasis in alkaliphiles have been characterized (Horikoshi, 1999). The *tupA* gene was identified in the *B. halodurans* genome, which is responsible for the synthesis of teichuronopeptide, a major structural component in the cell wall important for maintaining pH homeostasis (Takami et al., 1999). Alkylphosphonate

ABC transporter genes coding for two permeases, one phosphonate-binding protein and one ATP-binding protein, are the most frequent class of protein coding gene expressed in alkaliphiles. These transporters couple the hydrolysis of ATP to solute transport (Takami et al., 1999).

1.5.4. Proteome

Most proteins from alkaliphiles are extracellular enzymes. Comparative studies along with experimental and theoretical analysis have led to three main conclusions (Siddiqui and Thomas, 2008). First, the pK_a modulation of a catalytic residue toward higher pH is responsible for alkaline protein stability. This is achieved through modification of the hydrogen bonds and reduction in solvent exposure of the catalytic residue. Second, an increase in the surface exposure of acidic residues with respect to basic residues changes the net charge of the molecule toward negative. GH10 alkaline xylanase BSX from *Bacillus* sp. NG-27 and alkaline phosphoserine aminotransferase from *Bacillus alkalophilus* show such a trend (Siddiqui and Thomas, 2008). Third, the gain of glutamate plus arginine residues and the loss of aspartate plus lysine residues are key players in alkaline adaptation of proteins. Moreover, during the adaptation process it was observed that smaller hydrophobic residues were gained and larger ones lost when enzymes from alkalophiles and nonalkalophiles were compared (Siddiqui and Thomas, 2008). Modification of the proteome by increasing the fraction of acidic amino acids and reducing the protein hydrophobicity for alkaline stability has been observed in the haloalkaliphilic archaeon *Natronomonas pharaonis* (Horikoshi, 1998).

1.6. MOLECULAR EVOLUTION OF ACIDOPHILES

1.6.1. Habitat

Acidic environments are generally formed by natural processes. In such environments, ferrous iron and reduced forms of sulfur are often very abundant; thus, acidic environments are rich in sulfur (Nancucheo and Johnson, 2010). To a certain extent, anthropogenic activities contribute in the creation of acidic and metal-polluted milieus. In such environments, microbial aerobic and anaerobic metabolism generate acidity by the formation of inorganic acids, which results in most of the acidic environments. Nitrification and sulfur oxidation by microorganisms are potent contributors in the generation of such environments (Johnson et al., 2009). Elemental sulfur and sulfide minerals are oxidized to sulfuric acid by acidophilic bacteria and archaea in geothermal areas and in mine environments. Geothermal sulfur-rich sites known as *solfataras* are home to a variety of acidophiles (Johnson et al., 2009); for example, the solfatara fields in Yellowstone National Park, located near the Norris Geyser Basin, and at Sylvan Springs.

1.6.2. Cellular Organization

Acidophiles maintain a circumneutral intracellular pH (Baker-Austin and Dopson, 2007) by membrane impermeability to protons by the presence of tetraether lipids (Apel et al., 1980). Ether linkages characteristic of acidophilic membranes are less prone than ester linkages

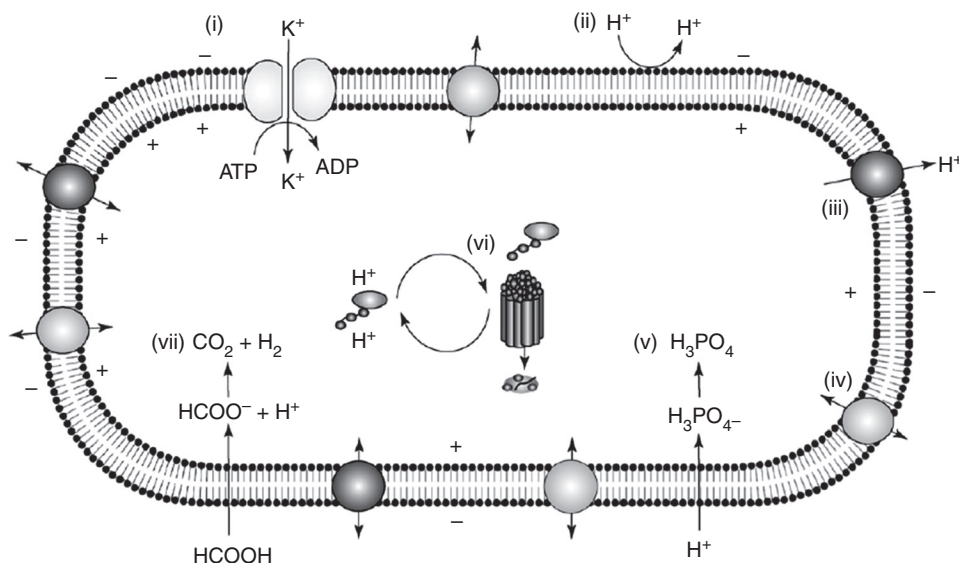


Figure 1.1. Processes associated with pH homeostasis in acidophiles. (i) Reversal of charge distribution occurs to stop the inward flow of protons through potassium-transporting ATPases. (ii) Impermeability of cell membranes to stop proton influx. (iii) Active proton export by transporters. (iv) Secondary transporters reduce energy demands of importing nutrients into the cell. (v) Certain enzymes bind and sequester protons. (vi) DNA and protein repair systems. (vii) Uncoupling of organic acids. [From Baker-Austin and Dopson (2007), with permission from Elsevier. Copyright © 2007.]

to acid hydrolysis (Golyshina and Timmis, 2005). Membrane channel proteins show a reduction in size (Amaro et al., 1991). An intracellular chemiosmotic gradient is created by the Donnan potential by positively charged molecules (Baker-Austin and Dopson, 2007). The presence of proton efflux protein systems is another interesting evolutionary feature (Tyson et al., 2004). Cytoplasmic buffer molecules having basic amino acids are capable of sequestering protons, hence maintaining pH homeostasis. For example, the glutamate and arginine are decarboxylated in *Escherichia coli*, resulting in cell buffering by proton consumption (Baker-Austin and Dopson, 2007). The overall process of pH homeostasis can be explained stepwise and is illustrated in Figure 1.1.

1.6.3. Genome

The genome size of acidophiles is smaller than that of neutrophiles. The smallest genome belongs to Thermoplasmatales (<2 Mb). Acidophiles possess genes for an organic acid degradation pathway (Angelov and Liebl, 2006). In extreme acidophilic genomes, functional characterization of a large number of DNA and protein repair genes (e.g., chaperones) gives a clue about their mechanism of acid homeostasis (Baker-Austin and Dopson, 2007). The genomes of acidophiles also contain a large number of pyrimidine codons, which are less susceptible to acid hydrolysis for protection from acid stress (Baker-Austin and Dopson, 2007; Paul et al., 2008). Tyson et al. (2004) also reported a variety of genes

involved in their unique cell membrane biosynthesis, indicative of a complex structure in microorganisms' acid tolerance capacity. Comparative genome analysis suggests that the acidophilic genome sequences show the presence of a higher proportion of secondary transporters and a larger proportion of DNA and protein repair systems than in neutrophiles, such as in the genome of the acidophile *Picrophilus torridus* (Baker-Austin and Dopson, 2007). Purines undergo acid hydrolysis. Thus, the genomes of thermoacidophiles such as *P. torridus* have evolved by lowering the purine-containing codons in long open reading frames (Baker-Austin and Dopson, 2007).

1.6.4. Proteome

Proteins of acidophiles were observed to be rich in acidic residues, which also show low solvent exposure. Stability is also obtained by replacement of charged amino acids by neutral polar amino acids in proteins reduces the electrostatic repulsion that occurs between charged groups at low pH (Norris, 2001). The presence of a high proportion of iron proteins contributes to acidic pH stability, as iron maintains the secondary structure of proteins at acidic pH by functioning as an "iron rivet" (Baker-Austin and Dopson, 2007). Chaperones involved in protein refolding are expressed strongly in acidophiles (Baker-Austin and Dopson, 2007). An increase in the isoleucine content of the proteins in acidophiles such as *P. torridus* was another trend observed, which was assumed to contribute to acid stability (Baker-Austin and Dopson, 2007). Another report by Settembre et al. (2004) brings forth the fact that an increase in the intersubunit hydrogen-bonding number of arginine-containing salt bridges in *Acetobacter aceti* PurE enzyme accounts for its increased acid stability.

1.7. MOLECULAR EVOLUTION OF BAROPHILES

1.7.1. Habitat

Barophiles are defined as those organisms displaying optimal growth at pressures above 40 MPa, whereas barotolerant bacteria display optimal growth at pressures below 40 MPa and can grow well at atmospheric pressure (Horikoshi, 1998). Aquatic environments with high pressure accompanied by low temperature are home to barophiles. The bottom of the deep sea is a world exposed to extremely high pressure and low temperature (1 to 2°C). But in the vicinity of hydrothermal vents, the temperature can raise to 400°C (Horikoshi, 1998), which is another temperature extreme of barophiles. Hydrostatic pressure increases at a rate of 10.5 kPa per meter of depth in the sea and decreases with altitude, and the boiling point of water increases with pressure, keeping it in the liquid state at the bottom of the sea. Thus, increased pressure increases the optimal temperature for microbial growth. Such microorganisms need to adapt to pressure challenges which cause volume change and a decrease in membrane fluidity (Rothschild and Mancinelli, 2001).

1.7.2. Cellular Organization

An increase in pressure leads to tighter packing of the molecules in lipid membranes, decreasing membrane fluidity (Rothschild and Mancinelli, 2001). Barophiles circumvent

such a situation, as they have evolved tightly packed lipid membranes with high levels of unsaturated fatty acids (Lauro and Bartlett, 2007) (e.g., docosahexaenoic acid) with low-melting-point lipids (Yano et al., 1998). Fungal strains such as *Graphium* species isolated from deep-sea calcareous sediment were reported to show microconidiation or the absence of hyphal growth (Raghukumar and Damare, 2008). In cultures of *Aspergillus ustus* under an elevated pressure of 100 bar, the hyphae showed thick swellings and beaded structures. Moreover, *Rhodospiridium sphaerocarpum* was reported to show multiple germ tube formation in yeasts (Raghukumar and Damare, 2008).

1.7.3. Genome

Survival at high pressure requires robust DNA repair systems (Rothschild and Mancinelli, 2001). Pressure-regulated operons have evolved in barophiles (Kato et al., 1995, 1996). Bartlett et al. (1989) discovered pressure-regulated promoter (*ompH*) and two open reading frames (ORF1 and ORF2) in *Photobacterium* sp. SS9. The promoter is activated at high pressure. Another pressure-regulated operon, designated ORF3, was identified in bacterial strain DSS12, which encodes for cytochrome *d* dehydrogenase (CydD) protein, which is required for the assembly of cytochrome *bd* complex and may be important for barophily (Kato et al., 1997; Horikoshi, 1998). Another such promoter was screened from the barophilic bacterium DB6705, which controlled chlorempenicol acetyl transferase gene expression at moderate pressure in *Escherichia coli* (Kato et al., 1997). It has also been reported that transcriptional efficiency of various ribosomal proteins is responsible for high-pressure adaptations in barophiles (Nakasone, 2005). Lauro and Bartlett (2007) reported that in barophiles, elongated helices occur in the 16S rRNA genes and their frequency increases with increased pressure. These helix changes are correlated with improved ribosome function under high-pressure conditions. Pressure-regulated *ompH* and *ompL* gene expression was studied through transposon and gene replacement mutagenesis experiments in *Photobacterium* sp. SS9. *ompH* was found to be necessary for a greater range of nutrient uptake at high pressure than was *ompL* (Horikoshi, 1998).

1.7.4. Proteome

Research work carried out in 2005 provided important insights into the role of amino acids in rendering proteins stable at high pressure and concluded that polar and small amino acids contribute more to barophilicity. These two amino acid properties are important for the origin of universal genetic code and support the hypothesis that genetic code structuring took place under high hydrostatic pressure (Giulio, 2005). The presence of proteins related to the heat-shock proteins in barophiles such as *Thermus barophilus* also aid in survival at elevated pressures (Marteinsson et al., 1999). Protease from *Methanococcus jannaschii* was the first enzyme to be characterized from a barophile (Horikoshi, 1998). This enzyme was reported to have narrow substrate specificity and higher activity at elevated pressure than under atmospheric pressure conditions (Horikoshi, 1998). Certain membrane proteins, such as the ToxR and ToxS proteins in *Photobacterium* sp. strain SS9, were also studied to assist in pressure adaptation controlling gene expression by the *oomph/ompL* pressure-regulated operons (Fig. 1.2) (Horikoshi, 1998). The enzymes of extreme barophiles are often folded differently as an evolutionary strategy under high pressure. Mutational studies by Horikoshii and others suggested that tryptophan uptake and trehalose accumulation in

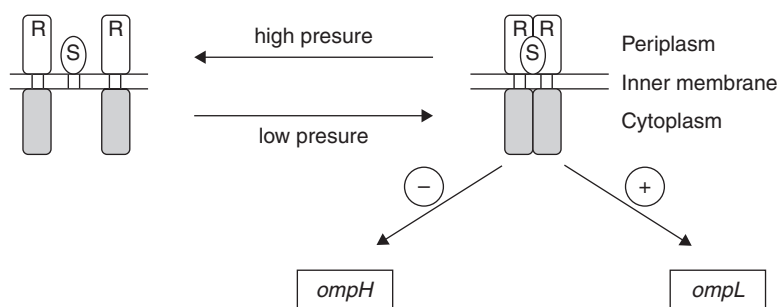


Figure 1.2. Model of Tox R/S function in the regulation of *omp* gene expression. [From Kato and Bartlett (1997), with permission from Springer Science + Business Media. Copyright © 1997.]

Sacharomyces cerevisiae are the key processes for survival under hydrostatic pressure by preventing the formation of protein aggregates (Abe et al., 2008).

1.8. ENGINEERING EXTREMOPHILES

Ground-breaking research on discovering extremophiles is based on their potential for industrial and biomedical applications (Hough and Danson, 1999; Cavicchioli and Thomas, 2000). However, extremophiles present a number of challenges for the development of bioprocesses because of their slow growth and low yield (Ludlow and Clark, 1991). These extreme conditions for culturing extremophiles are also incompatible with standard industrial fermentation and downstream processing equipment (Gomes and Steiner, 2004). “Microbes are available now but they are not effective for the most part,” says marine microbiologist Jay Grimes of the University of Southern Mississippi (Biello, 2010). Therefore, a crucial goal in this field is determining the features that are critical for their extremophily so that engineering extremophiles will be possible. To engineer extremophiles it is of prime importance to understand their physiology and biochemistry. Knowledge of experimental evolution is one way for naturally engineered extremophiles to modify their metabolic pathways as well as to optimize growth rates. This can be accomplished using genomic informatics, genetic engineering, and directed evolution strategies. The methods are described briefly in the following sections.

1.8.1. Microbiology

Manipulation of the milieu of extremophiles through selection pressure to make them robust is one of the oldest approaches. Routine microbial techniques have been employed to enhance extremophilicity. The organisms are grown in minimal or auxotrophic media, subjected to various combinations of selection pressure (e.g., heat, pressure, temperature), or to physical and chemical mutagens such as ultraviolet irradiation and hydroxylamine, respectively. Chemical mutagens such as hydroxylamine cause DNA damage and change in base pairing by tautomeric shift. Briefly, the method of engineering extremophiles involves growing cells in a culture medium to the early exponential phase and subjecting them to centrifugation. The pellets are then resuspended in a fresh culture medium and

transferred to tubes containing a different concentration of the chemical mutagen. To stop the reaction the cells are again centrifuged and washed with a salty solution (Rodríguez-Valera et al., 1980). The washed pellets are then suspended in a liquid medium and grown overnight. The appropriate dilution is selected and the mixture is plated in a solidified medium. For example, hydroxylamine has been used to mutate halophiles of the family Halomonadaceae (Vargas and Nieto, 2004). Llamas et al. (1999) mutated *Halomonas eu-rihalina* using hydroxylamine and obtained nonmucoid mutants of its F2-7 strain which can be used as a genetic tool to discover and study the genetic determinants of bacterial exopolysaccharides. The surviving colonies were then screened for any changes, and experiments were performed regularly to study interesting developments in the population. Microbial techniques such as vertical and horizontal gene transfer through conjugation and protoplast fusion can be employed to engineer extremophiles. An interesting approach in this regard, which has been patented, involves plasmid transfer by mating of *Pyrococcus furiosus* with *E. coli*. The method involves interkingdom gene transfer by cocultivating an isolated recipient extremophile and a member of the family Enterobacteriaceae. Further steps involve identifying an exconjugant that includes at least a portion of the conjugative gene that has been introduced, integrated into its genomic DNA (Michael et al., 2009). Horizontal gene transfer has also been used successfully to transfer vectors from *E. coli* to halophiles such as *Chromohalobacter*, *Halomonas*, and *Salinivibrio* (Vargas and Nieto, 2004).

1.8.2. Molecular Biology

Recombinant extremophiles can be evolved in vitro by incorporation of foreign genes into the native genome. The foreign gene required can be inserted in the host strain following molecular biology protocols. The first step involves cloning the desired recombinant DNA in large copy numbers, integrating vector plasmids lacking an origin of replication for archaea or shuttle vector systems possessing the origin of replication indigenous to the host (Allers and Mevarech, 2005). The gene–vector construct can also be transformed into an extremophile through biolistics, polyethylene glycol, and transposon-mediated integration of plasmid containing the gene(s) of interest, depending on the target extremophile. For example, Cline and others performed polyethylene glycol–mediated transfection of *Halobacterium halobium* with naked DNA from phage FH; this method of transfection has been adapted for other archaea, such as *Methanococcus maripaludis* and *Pyrococcus abyssi* (Cline and Doolittle, 1992; Allers and Mevarech, 2005). However, one drawback of all the methods described above is that it is only effective in species for which spheroplast can be generated. Similarly, electroporation can be used for *Methanococcus voltae* but not for *Methanosarcina acetivorans*. To overcome this problem, autonomously replicating plasmid vectors for such extremophiles as *Thermus thermophilus* have been constructed by several groups using trpB, β -galactosidase, or kanamycin resistance genes as selectable markers (Tamakoshi et al., 1997). Moreover, shuttle integration vector systems have also been developed which can integrate in the extremophilic genome by homologous recombination and can also be recovered from recombinant hosts. One major problem in cloning genes in extremophiles such as *Haloferax volcanii* is that they have a restriction system that recognizes adenine-methylated GATC sites frequently found in vectors that are based on *E. coli* plasmids, resulting in DNA fragmentation followed by plasmid loss (Blaseio and Pfeifer, 1990; Allers and Mevarech, 2005). This can be overcome by cloning the DNA first

in an *E. coli* *dam*⁻ strain, which is deficient in GATC methylation (Holmes et al., 1991; Allers and Mevarech, 2005). The next step is selection of recombinant clones. In the case of extremophiles, puromycin and novomycin are used routinely as selectable markers. One problem associated with such markers is that their genes are prone to instability, owing to homologous recombination. This problem can be overcome using marker genes in vectors from distantly related species.

An indirect and much simpler way than genetic modification of engineering extremophiles is by expressing their targeted genes in recombinant hosts. The first step in this regard would be cloning genomic DNA from extremophiles. Several commercially available kits can also be used for this purpose. The second step is molecular library creation and screening. This is achieved through DNA fragmentation with restriction enzymes and further cloning in vectors such as pBR322 (Mellado et al., 1995). As genomes of a number of extremophiles have already been sequenced and characterized, the genome size of a particular strain can be assessed, and thus a complete genome can be successfully cloned into a library. One well-known vector system is the pET series from Novagen. Construction of genomic libraries with bacteriophages leads to high-quality libraries of extremophilic genomes. The third step involves the expression of desired products such as enzymes in a recombinant host such as *E. coli* strains or Halomonadaceae (Vargas and Nieto, 2004). An example of such an approach is the expression of ice-nucleation protein from *Pseudomonas syringae* and amylases from *Pyrococcus woesei* in halophiles using native or heterologous promoters (Vargas and Nieto, 2004).

Another approach to genetically engineered extremophiles is through gene-knockout strategies. Extremophiles are transformed with the construct for gene knockout using circular DNA, which is more stable than linear DNA fragments, and selection of the recombinant host is achieved most efficiently through reusable uracil auxotrophic counter-selectable marker systems (Allers and Mevarech, 2005). Details of this strategy are presented in Figure 1.3. One problem is that Gelrite, used in solid media for culturing hyperthermophiles, contains trace amounts of uracil. Another drawback is the failure of the gene construct to recombine with the host chromosome. Thus, the selection of strains proficient in homologous recombination can overcome such problems (Worthington et al., 2003; Allers and Mevarech, 2005). One example where gene-knockout strategy was employed successfully to generate genetically modified extremophiles was through complete replacement of the *leuB* gene with the *pyrE* gene and further deletion of the *pyrE* gene by using 5-fluoroorotic acid in the host strain, *Thermus thermophilus* (Tamakoshi et al., 1997). The results of such an interesting effort supports the assumption that this gene-knockout strategy is useful for the reconstruction of a reliable plasmid vector system and that it can be used in the selection of stabilized enzymes (Tamakoshi et al., 1997).

Directed evolution is another molecular biology strategy used to evolve extremophilic native enzymes in the laboratory by applying a directional approach for emulating the enzyme. Based on the blueprints of well-characterized extremophilic proteins, unknown proteins lacking those stability properties can be engineered by directed evolution experiments which comprise random mutagenesis steps using physical (UV irradiation) or chemical mutagens or by error-prone polymerase chain reaction (PCR), recombination, and screening processes. Directed evolution was performed on a psychrophilic enzyme, subtilisin S41 (Davail et al., 1994), to increase its thermostability (Miyazaki et al., 2000; Wintrod et al., 2001). Raffaele et al. (2001) characterized a mutated version of the hygromycin B phosphotransferase gene from *Escherichia coli*, isolated by directed evolution

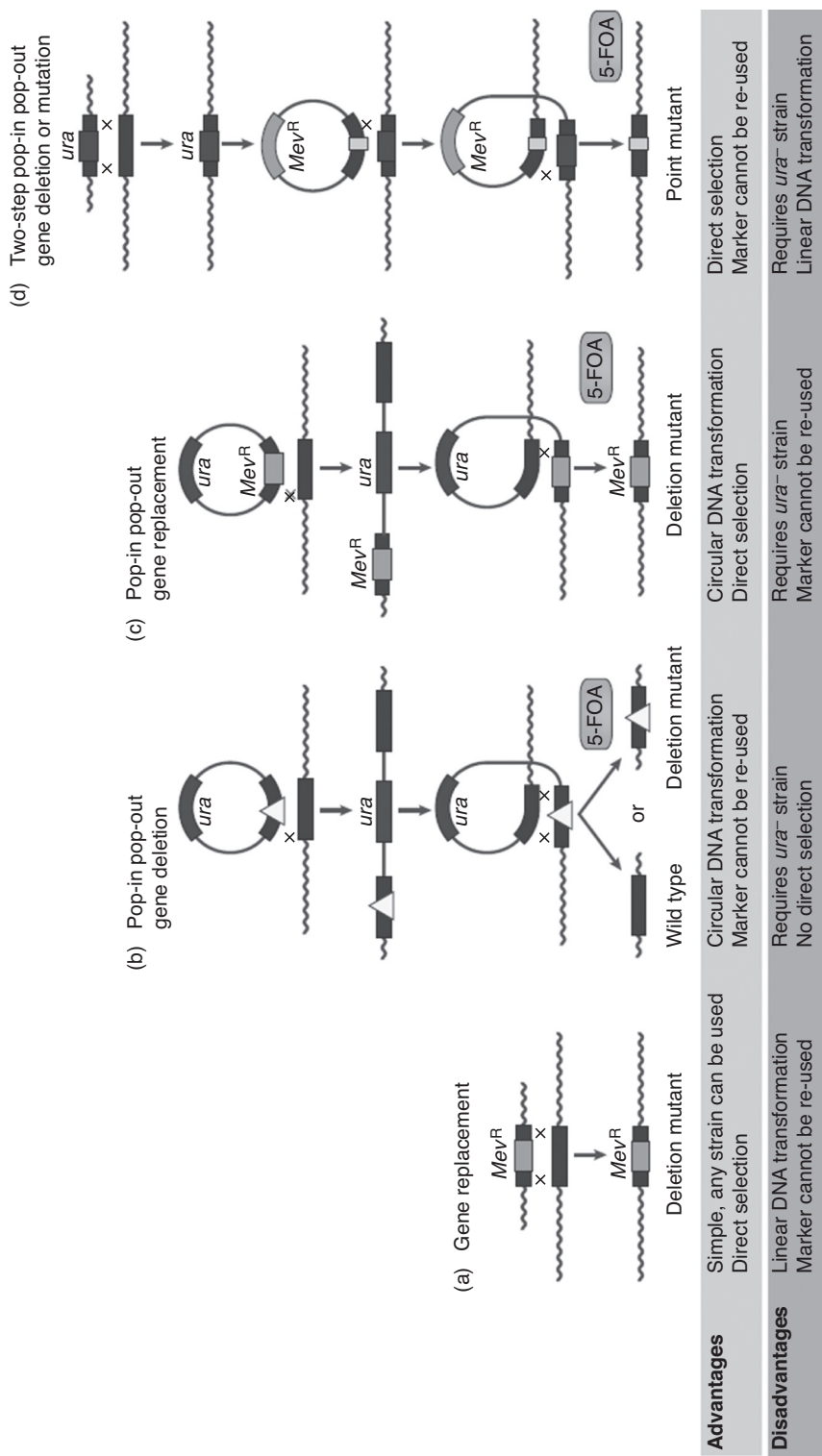


Figure 1.3. Gene-knockout methods used in archaeal genetics. (a) Direct replacement of a gene with a selectable marker, by recombination between linear DNA, which comprises flanking regions of the gene, and a chromosomal target. (b) The pop-in pop-out method uses circular DNA and selection for transformation to uracil prototrophy. (c) Variant of the pop-in pop-out method for gene deletion, in which the gene is replaced by a marker that allows direct selection. (d) Combination of gene replacement (with *ura* marker) and the pop-in pop-out method, suitable for generating point mutations. [From Allers and Mevarech (2005), with permission from Macmillan Publishers Ltd. Copyright © 2005.]

in transformants of *Sulfolobus solfataricus* with respect to its genetic stability in both the original mesophilic and the new thermophilic hosts. One drawback of the method is that it is time consuming, requiring several generations of mutagenesis recombination and screening. However, random mutagenesis employed for directed evolution strategy is time consuming, owing to generations of mutagenesis recombination and screening. In directed evolution a specific property is subjected to selection pressure. Thus, a site-directed mutagenesis technique of directed evolution is gaining popularity among researchers worldwide. The procedure involves the synthesis of a short DNA primer containing the desired base-change mutation subjected to PCR with the target DNA of interest in plasmid. After a sufficient number of PCR cycles, the mutated fragment will be amplified sufficiently with respect to the unmutated plasmid by gel electrophoresis.

1.8.3. Bioinformatics

Functional genomics and genomic informatics can be used to manipulate an organism's biology (Daly, 2001). A significant number of genomes from extremophiles have been sequenced and deposited in databases such as the NCBI (National Centre for Biotechnology Information). Complete genome sequencing of *Methanococcus jannaschii*, *Colwellia psychrerythraea*, *Thermoplasma acidophilum*, and *Sulfolobus acidocaldarius* are a few such examples. From this vast catalog of information about the open reading frame of interest, first, knowledge of their in vitro/in vivo manipulation can be gained. Such knowledge can lead to expression of novel gene products in recombinant host strains. Second, multiple alignments of extremophilic protein sequences through servers such as ClustalW, which can be accessed through <http://www.ebi.ac.uk/Tools/msa/clustalw2/>, can aid in the design of degenerate PCR primers to fish out genes from newly isolated organisms. Degenerate primers are mixtures of similar primers. They are used when homologous genes are to be amplified from uncharacterized genomes from newly isolated organisms or genomes isolated from the environment through metagenomic approaches. Rose et al. (2003) developed specific degenerate primers known as CODEHOPs (Consensus Degenerate Hybrid Oligonucleotide Primers). A CODEHOP is degenerate at the 3' core region and is non-degenerate at the 5' consensus clamp region. The workflow of designing CODEHOPs is represented schematically in Figure 1.4.

CODEHOPs have been used to fish out new genes from extremophiles. For example, Han et al. (2010) used CODEHOP PCR to clone selected regions of the *phaE* and *phaC* genes' haloarchae, encoding PHA synthases (type III), which were sequenced further. Moreover, structural analysis of proteins from extremophiles through homology modeling, molecular superimposition, and molecular docking can guide in the molecular design of their properties in homologous nonextremophilic counterparts by site-directed mutagenesis and directed evolution strategies and thus lead to their in vitro evolution. Moreover, extremophilic databases have been created which are freely accessible to the public. One database is CCCryo (Culture Collection of Cryophilic Algae), which can be accessed through <http://extrem.igib.res.in/>, created by the Institute of Genomics and Integrative Biology in India. The halophile genome site, which is a database of annotated halophilic genomes, can be accessed through <http://edwards.sdsu.edu/halophiles/>. The annotation was done by the Rapid Annotation Using Subsystems Technology (RAST) server, and the database can be accessed through <http://edwards.sdsu.edu/halophiles/>.

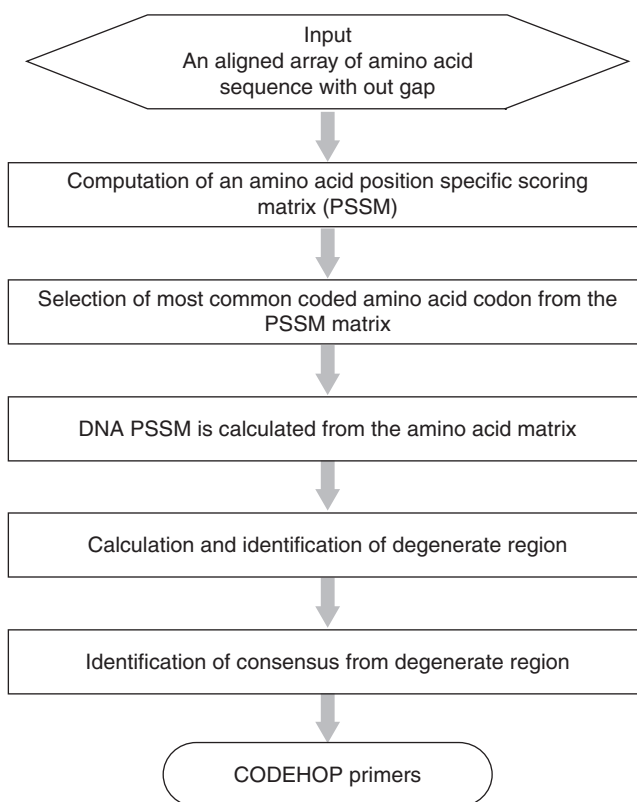


Figure 1.4. Flowchart showing the process of designing CODEHOPs. [Adapted from Rose et al. (1998), by permission of Oxford University Press.]

1.9. CASE STUDIES

1.9.1. Biofuel Production

A microbial strain with high ethanol tolerance and yield is a good candidate for biofuel production and an efficient extremophile. *Thermoanaerobacterium*, a heterofermentative thermophile, was genetically engineered for a single knockout mutant for lactate dehydrogenase in *Thermoanaerobacterium*, resulting in reduced levels of lactate production and a fourfold increase in ethanol production (Desai et al., 2004). *Clostridium thermocellum* is a good candidate for bioethanol production; however, the strain is sensitive to high ethanol concentration. Thus, a strain tolerant of high ethanol concentration was genetically engineered (Lynd et al., 2005). In other research, a *C. thermocellum* lactate dehydrogenase mutant, with higher ethanol production and enhanced product tolerance, was constructed through electrotransformation of a foreign gene for ethanol production (Tailliez et al., 1989). The UK-based company Green Biologics genetically modified extremophiles to produce butanol, which is commercially sold as Butafuel (Barnard et al., 2010). Xylose utilizing the thermophile *Thermoanaerobacterium saccharolyticum* was subjected to end-product

metabolic engineering for ethanol production, as thermophilic microbes isolated to date cannot produce ethanol in consistently high yields. Genes involved in lactate (*ldh*) and acetate (*ack/pta*) production were knocked out, resulting in a strain able to produce ethanol as the only detectable organic product (Shaw et al., 2008). A lactate dehydrogenase mutant, *Thermoanaerobacter* BG1L1, was engineered with resistance to 8.3% ethanol. This strain is also robust in utilizing xylose from lignocellulosic hydrolysates, stover pretreated with dilute acid, and wheat straw hydrolysate (Georgieva et al., 2007).

1.9.2. Bioremediation

Bioremediation is the use of biological agents to reduce toxic substances in the environment to a nontoxic or less toxic state. The most radioactivity-resistant organism, *Deinococcus radiodurans*, has been genetically engineered for use in bioremediation in oil, heavy metal, and mixed radioactive metal contaminations. It is robust because of its unique capability to repair damaged DNA (Clark et al., 2009). Mercuric reductase gene (*mer A*) has been cloned from *E. coli* strain BL308 into *Deinococcus* to detoxify the ionic mercury residue found in radioactive waste generated from nuclear weapons manufacture (Brim et al., 2000). Moreover, a recombinant strain of *Deinococcus radiodurans* which degrades organopollutants under radioactive conditions has been engineered by cloning toluene dioxygenase from *Pseudomonas putida* F1 into its genome, enabling it to oxidize toluene, chlorobenzene, and indole in a highly irradiating environment. An added advantage of the strain is that it remains tolerant of trichloroethylene at appreciable levels (Lange et al., 1998). The only disadvantage is that it cannot operate in the thermophilic range; thus, *Deinococcus geothermalis* was transformed genetically with plasmids designed for *Deinococcus radiodurans*. This made it possible to reduce Hg(II) at elevated temperatures in the presence of 50 Gy/h (Brim et al., 2000).

1.9.3. Pesticide Biodegradation

Pesticides persist in the environment for a long time without undergoing biological transformation. Extremophilic microorganisms are a potent source for degrading such xenobiotics, as they are adapted to grow and thrive in such environments. Thermophiles such as *Pseudomonas* spp. are good candidates for pesticide bioremediation (Margesin and Schinner, 2001). However, the limitation is that a single microbial species is specific for a particular xenobiotic compound. Pesticide bioremediation increased dramatically following characterization and cloning of pesticide-degrading genes from such microorganisms. A pioneering work in this field was the creation of a superbug from genetically engineered *Pseudomonas putida* by Ananda Mohan Chakrabarty at General Electric in 1975. The genetically engineered *P. putida* was potent in degrading camphor, naphthalene, xylene, toluene, octanes, and hexanes (Chakrabarty, 1976). The genes responsible for the degradation of octane, xylene, camphor, and naphthalene were cloned from different plasmids of various microbial strains and transformed into a single cell of *P. putida*. The plasmids of recombinant *P. putida* degrading various chemical compounds are TOL (for toluene and xylene), RA500 (for 3,5-xylene), pAC 25 (for 3-chlorobenzoate), and pKF439 (for salicylate toluene). Researchers have also transformed *Pseudomonas* with two genes responsible for Y-HCH or lindane degradation present in halogenated pesticides: *linA* and

linB (Nagata et al., 1994). These genes are a member of the haloalkanedehalogenase family with a broad substrate specificity range. Another pioneering work in this field relates to a halophile, *Halobacterium* sp., subjected to a patented selection process of environmental adaptation to high concentrations of various xenobiotics, such as phenolics and aromatic compounds (Oesterhelt et al., 1998).

1.9.4. *Escherichia coli*: A Candidate Extremophile

E. coli has been the most favored workhorse for genetic engineering with the introduction of genes from extremophiles for stable expression of enzymes for industrial and biomedical applications. For example, *E. coli* is unable to produce butanol naturally, thus has been engineered with the introduction of genes from extremophiles for the production of higher-chain alcohols. The plasmid WWO of *P. putida*, which is useful for hydrocarbon degradation, has been propagated in *E. coli* (Franklin et al., 1981). Another example is the citramalate synthase enzyme from *Methanococcus jannaschii*, which was overexpressed in *E. coli* for the production of both 1-propanol and 1-butanol (Atsumi and Liao, 2008). Researchers also attempted to induce radioactive resistance in *E. coli* by directed evolution (Clark et al., 2009). In 2011, directed evolution strategy was employed to educate the model bacterium *E. coli* to develop the ability to survive exposure to high temperature or pressure (Vanlinta et al., 2011). Recent research in Japan subjected *E. coli* to an extreme of gravity. The bacteria were cultured after being rotated in an ultracentrifuge at a high speed: $403,627 \times g$. Analysis showed that the small size of prokaryotic cells is essential for successful growth under hypergravity. This research implicates the feasibility of panspermia (Deguchi et al., 2011).

1.9.5. Oil-Spill-Cleaning Bacteria

Many halophiles have the unique ability to degrade specific hydrocarbons in oil to access energy, but oil spills contain a mixed variety of hydrocarbons. Thus, it is necessary to engineer extremophiles to make them robust for degrading more than one type of hydrocarbon. *Alcanivorax borkumensis*, which can decompose hydrocarbons aerobically, is a blueprint organism for genetic modification for the same purpose. Evolugate, a Florida company, developed microbes for bioremediation of oil spills. Microbes are grown in continuous cell culture vessels under selection pressure to increase cell proliferation (Dimond, 2010). Such extremophiles can therefore be engineered for degrading high-molecular-weight paraffins, asphaltenes, and sulfur heterocycles (Seckbach, 2000).

1.9.6. Potential Applications and Benefits

The potential use of genetically engineered extremophilic microorganisms is an important and exciting prospect. They are used in bioremediation, medicine, and commercial products such as detergents. *Deinococcus radiodurans* is being engineered for the remediation of radioactive waste (Brim et al., 2000). Engineered extremophiles build up revenues for companies. For example, Genencor, a profitable biotechnology company, has the genetic material of 15,000 strains of microbes stored in deep-freeze in Palo Alto, California, and the Netherlands. It is using alkaline resistance genes from alkaliphiles in East Africa and Kenya

to create enzymes for laundry detergent. For example, enzymes from alkaliphiles have been used in Tide detergent and to give jeans a faded look. In the field of medicine a eukaryotic homolog of the *myc* oncogene product from halophilic archaeae has been used to screen the sera of cancer patients. The archaeal homolog produced a higher number of positive reactions than the recombinant protein expressed in *Escherichia coli* (Satyanarayana et al., 2005). Interestingly, the Defense Advanced Research Projects Agency in the United States is sponsoring experiments on genetically engineering extremophiles to extend the shelf life of blood-clotting platelets in extreme conditions to treat battlefield wounds. The ultimate effort in this field was made by the Craig Venter Institute, which produced a synthetic organism, *Mycoplasma laboratorium* (Reich, 2000). An article in the *Scientific American* by David Biello in 2010 stated that “the first microbe to live entirely by genetic code synthesized by humans has started proliferating at a lab in the J. Craig Venter Institute.” Moreover, the government of Kuwait is using engineered extremophiles to clean up gallons of spilled oil.

1.10. IMPLICATIONS OF ENGINEERED EXTREMOPHILES ON ECOLOGY, ENVIRONMENT, AND HEALTH

Whether engineered extremophiles are a boon or a bane to ecology and health is a hot topic of discussion among scientific communities worldwide. On the negative side, once genetically modified extremophiles (GMEs) are introduced into the environment it may be impossible to eliminate them. Their persistence in the milieu is almost equivalent to that of wild-type organisms. GMEs can also lead to elimination of a wild-type microbial species due to competition. Horizontal gene transfer to wild-type and pathogenic organisms can cause adverse effects. They can also be opportunistic pathogens in immunosuppressed hosts. On the other hand, they may be targeted for development as biowarfare agents (Daly, 2001).

Advantages of GMEs include their use as sources of enzymes and other molecules for diagnostic and pharmaceutical purposes (Irwin and Baird, 2004). A good example is that extremophile extracts such as the iron-binding antifungal compound pyochelin from *Pseudomonas* spp. were found to have positive activity against pathogenic *Candida* and *Aspergillus* spp. (Phoebe et al., 2001). In the food industry, dried *Dunaliella* (halophile) has shown potential as a food supplement with antioxidant properties (Irwin and Baird, 2004).

1.11. CONCLUSIONS AND RECOMMENDATIONS

The evolutionary mileage of the various classes of extremophiles are specific for their subtypes and not accounted for in neutrophiles, indicating the role of evolution in determining precise and highly specific molecular mechanisms for extreme adaptations. The evolution of extremophiles can be summarized, emphasizing adaptations in cell membranes, metabolic pathways, genome, and proteome. Important differentiation landmarks are observed in the phospholipid bilayer of thermophiles and acidophiles, which is composed of ether linkages, compared to ester linkages in neutrophiles. The compactness of the cell membrane in psychrophiles and barophiles, with its high levels of unsaturated fatty acids, and the presence of phosphatidyl glycerol sulfate in halophiles can be cited as their evolutionary trademarks.

Natural metabolic pathway engineering is observed in thermophiles and psychrophiles, wherein the synthesis pathway of heme, acetyl-CoA, acyl-CoA, and folic acid and the pathway for formation of reactive oxygen intermediates are eliminated. Pertaining to the genome of extremophiles, an important evolutionary marker is the high GC content of thermophilic, psychrophilic, and halophilic genomes. The presence of reverse gyrase in thermophiles, nucleic acid-binding proteins such as RNA helicases in psychrophiles, purine-rich codons in thermophilic and psychrophilic genomes, pyrimidine-rich tracts in acidophiles, robust DNA repair systems, and the presence of pressure-regulated operons in barophiles are some noteworthy exceptions and discoveries. On the proteome level, thermophiles and psychrophiles differ markedly from neutrophiles by synthesizing chaperones and antifreeze proteins, respectively, which leads to refolding of denatured proteins at such extremes. Bulky hydrophobic and charged residues show higher frequency in thermophilic proteins, whereas smaller ones are preferred in psychrophiles. Acidophiles and alkalophiles show a preference for acidic and small hydrophobic residues in their proteins.

Protein compactness and increased hydrogen bonding in thermophiles and psychrophiles are other noteworthy trends. In the secondary structure of extremophilic proteins, high α -helix and β -sheet content are observed in thermophiles, whereas halophilic proteins favor loops over helices as a mode of extreme adaptation. Despite the foregoing, extensive capital funding and concerted efforts are required to understand the diversity of extremophiles and hence their exploitation. The bottlenecks are that proper genetic systems for high-level expression are still rare for extremophiles, and although the complete genome sequences of thermophiles such as *Thermoplasma acidophilum* and *Sulfolobus acidocaldarius* have recently been published, they still lack global annotation. Culturing extremophiles in vitro is challenging, and researchers thinking of switching to archaea should ponder the fact that only a single model organism is available for this entire domain. We hope that our work will be helpful to researchers in engineering potent extremophiles.

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ATTAINING EXTREMOPHILES AND EXTREMOLYTES: METHODOLOGIES AND LIMITATIONS

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2.1. INTRODUCTION

It has been said that “Normal is passé; extreme is chic” (Rothschild and Mancinelli, 2001). *Extreme* is a relative term in comparison to what is normal for humans. A variety of microbes live in extreme conditions of temperature, pH, pressure, salt concentration, water availability, radiation, heavy metals, toxic compounds (organic solvents), and other unusual geological milieus. Such microorganisms were termed *extremophiles* by MacElroy in 1974 (Rothschild, 2007). Microorganisms that are able to survive in more than one extreme environment are known as polyextremophiles (Rothschild and Mancinelli, 2001). Extremophiles are assumed to have evolved around 1 billion years ago and were first discovered in the hot springs of Yellowstone National Park in the United States (Horikoshi, 2011). Research on extremophiles continues to increase, as exemplified by the recently discovered twenty-second genetically encoded amino acid, pyrrolysine, from the archaeon *Methanosarcina barkeri* (Srinivasan et al., 2002). An exact chronology cannot be assigned to the origin of all life relative to that of extremophiles, and the theories relating to extremophilic evolution are a subject of anthropocentrism. Martin and his co-workers (2008) published an article proposing that extremophiles originated in hydrothermal vents, where all life is believed to have had its genesis. Davies (1999) has furnished a variety of reasons to believe that life on Earth evolved near a thermal vent.

Extremophiles occupy the shorter branches of rRNA-based molecular phylogenies and paleontological records and at first glance lead to the idea of a hot origin of life (Islas et al., 2007). Figure 2.1 depicts the universal tree of life and the origin of extremophiles from the last common ancestor. On the other hand, through a complex Markov model, researchers have proposed that the universal ancestor was a mesophile, as high temperature could lead to denaturation of biochemical compounds which have half-lives for decomposition of just a few minutes at temperatures between 250 and 350°C (White, 1984; Miller and Bada, 1988). Furthermore, extremophiles employ complex enzyme-dependent mechanisms for the protection of their genome (Grogan, 1998), which is unlikely to have been true during prebiotic times. A more likely explanation would be that hyperthermophiles have evolved through secondary adaptations during early stages of cell division. Irrespective of the aforesaid, it is speculated that during the mid-Achaean era, a clade known as terrabacteria, comprised of extremophilic organisms, evolved. It is assumed that after a “hot” thermophilic beginning, acidophilic organisms evolved followed by alkaliphilic organisms when sufficient mineral precipitation occurred in the atmosphere. Lozupone and Knight (2007) concluded that salinity determined the composition of microbial community rather than temperature. The oldest fossils of life on Earth are found in microfossils and stromatolites. The earliest stromatolites were probably prephotosynthetic eubacteria ancestors of *Chloroflex* and *Chlorobium*, both photosynthetic archaean anaerobic thermophiles that preceded the cyanobacteria. It can be said conclusively that emergence of the first living organism is still a mystery, and the environmental conditions under which organisms can thrive should be considered as evidence of their adaptability and not as evidence that the origin of life took place under extreme conditions.

2.2. EXTREMOPHILES: TYPES AND DIVERSITY

Recent work suggests that extremophiles belong to a diverse group of organisms classified into nine categories based on their environmental niche, the majority of which remain uncultured (Hough and Danson, 1999). Extremophiles span all three domains of life. Hyperthermophiles dominate archaea and bacteria; psychrophiles, acidophiles, alkaliphiles, piezophiles, xerophiles, and halophiles also have eukaryotic siblings. Complicating the systemic classification further, extremophiles include multicellular organisms, and psychrophiles include vertebrates (Rothschild and Mancinelli, 2001). The major classes of extremophiles are summarized in Table 2.1.

Working with extremophiles requires special techniques and skills, due to the difficulties faced in culturing them because most of them remain uncultured. The isolation and characterization of six main classes—thermophiles, psychrophiles, halophiles, alkaliphiles, acidophiles, and barophiles—are discussed thoroughly with their associated technical limitations of culturing methodologies.

2.2.1. Thermophiles

Milieu. Thermophiles thrive at temperatures above 45°C in various geothermally heated regions of the Earth, such as hot springs, geysers, environments associated with high volcanic activity, deep-sea hydrothermal vents, and decaying living matter. Terrestrial hot springs are present in most geothermal areas in the United States, Iceland, New Zealand,

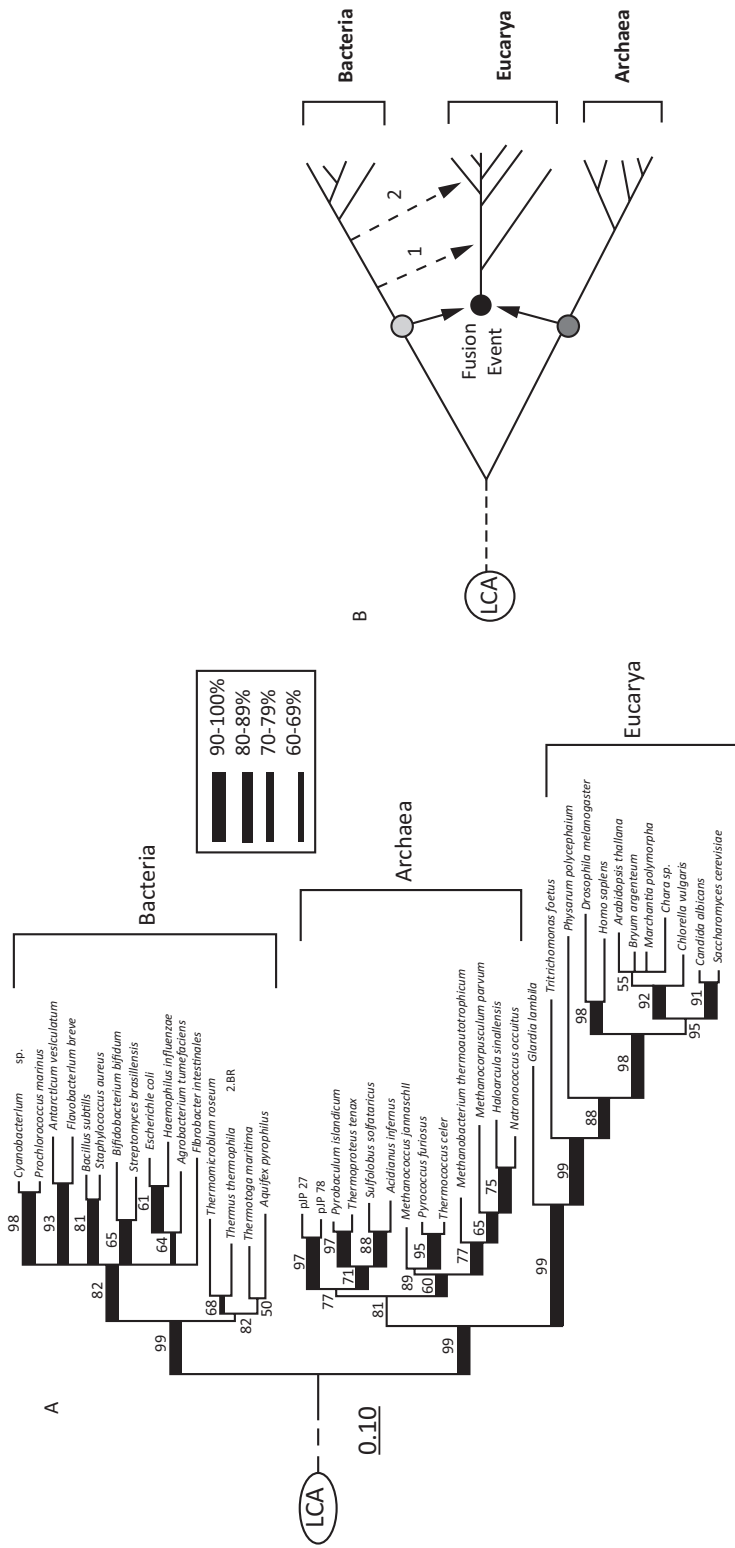


Figure 2.1. (A) The universal tree of life. (B) Origin of bacteria, archaea, and eukaryotes from the last common ancestor. [From Bhattacharya et al. (1999), with permission from Kluwer Academic.]

TABLE 2.1. Major Classes of Extremophiles

No.	Extreme Milieu	Class	Growth Conditions	Examples
1	Temperature	Hyperthermophile Thermophile Psychrophile/cryophile	> 80°C 60–80°C < 15°C	<i>Methanopyrus kandleri</i>
2	pH	Acidophile Alkaliphile	pH < 3 pH > 9	<i>Polaronas vacuolata</i> Cyanidium caldarium, <i>Picrophilus oshimae</i> <i>Spirulina</i> spp., <i>Natronobacterium gregoryi</i>
3	Salinity	Halophile	0.2 M concentrations of NaCl	<i>Dunaliella salina</i> , <i>Halobacterium salinarum</i>
4	Geological barriers	Cryptoendolith Hypolith Xerophile	Microscopic spaces within rocks Underneath rocks in cold deserts Extremely dry, desiccating conditions	<i>Ostreobium</i> sp. <i>Streptomyces hypolithicus</i> <i>Trichosporonoides nigrescens</i>
5	Radiation	Radioresistant	Resistant to high levels of ionizing radiation	<i>Deinococcus radiodurans</i>
6	Pressure	Piezophile	High hydrostatic pressure	Strain MT41
7	Chemical extreme	Metallotolerant	High levels of dissolved heavy metals in solution	<i>Cupriavidus metallidurans</i>
8	Osmotic barrier	Osmophile	High sugar concentration	<i>Saccharomyces rouxii</i>
9	Nutritional extreme	Lithoautotroph	Carbon is carbon dioxide and exergonic inorganic oxidation	<i>Nitrosomonas europaea</i>
10	Polyextremophiles	Oligotroph Thermoacidophile	Nutritionally limited environments Extremophiles falling under multiple categories	<i>Pelagibacter ubique</i> <i>Picrophilus oshimae</i>

Japan, India, Italy, Indonesia, Central America, Russia, and Central Africa (Madigan and Martino, 2006). High temperatures caused by biological activity of microorganisms are also a promising source of thermophiles, as in coal refuse piles (Beffa et al., 1996). They are also present in fermenting materials such as compost piles and silage, which can reach temperatures of 60 to 65°C. Additional thermal environments include human-made domestic and industrial hot-water systems and industrial high-temperature equipment (e.g., paper and food processing).

An environment that is still poorly explored for the presence of prokaryotic thermophiles is the deep subsurface of the Earth (Gold, 1992). Thermophiles have been found in soil that receives full sunlight, reaching 50°C at midday. But the most extreme thermophiles are found in environments near active volcanoes: terrestrial hot springs, solfatera fields (areas of fading volcanic activity whose soil excretes sulfuric gas), and deep-sea hot spring vents, commonly referred to as “black smokers” (Bhattacharya et al., 1999). The search for thermophiles in undersea volcanic areas has shown a wealth of organisms, all belonging to the domain Archaea, that can grow at temperatures higher than those of the boiling point of water at normal atmospheric pressure (Stetter, 1996). Even more challenging environments in which to search for hyperthermophiles are deep-sea hydrothermal vents: the anoxic sulfide and mineral-loaded waters emitted by black smoker chimneys, which can reach temperatures up to 350°C. A good example is an iron-reducing archaeon, still incompletely characterized, known as strain 121, that reportedly grows at 121°C, the temperature inside an autoclave (Kashefi and Lovley, 2003).

Biodiversity. Thermophilic bacteria were discovered in 1960 (Brock, 1994) when Brock isolated *Thermus aquaticus* from Mushroom Spring in Yellowstone National Park. However, only a small portion of the microorganisms have been characterized (Reysenbach and Shock, 2002). Phylogenetically, they belong to bacteria and archaea (Stetter, 1999). Figure 2.2 illustrates the distribution of hyperthermophiles among bacteria and archaea. In 2002, Reysenbach and Shock reported that hydrothermal milieu with temperature between 50 and 90°C are dominated by bacteria. In hot springs with a temperature range of 60 to 68°C, *Hydrogenobacter* are dominant. Thermophilic fungi have been found in anthropogenic, self-heated habitats (Tansey and Brock, 1971). Highly thermophilic niches with temperatures above 90°C are dominated by archaea (Reysenbach and Shock, 2002). However, extreme thermophiles, those surviving above 65°C, comprise prokaryotes only (Madigan et al., 2003).

Species composition varies in different thermophilic niches, with one or two dominating species. For example, bacterial species belonging to *Aquifae*, *Proteobacteria*, *Cyanobacteria*, and *Chloroflexi* dominate Iceland geothermal hot spots. *Thermoproteus*, *Thermophilum*, and *Desulphorococcus*, anaerobic archaea and bacteria that have a temperature optimum of 85 to 95°C, are found in clayish hot springs (Vesteinsdottir, 2008). In hot springs with low and high sulfide concentrations, *Chloroflexus* and *Aquificales* dominate, respectively (Skirnisdottir et al., 2000). The diversity of thermophiles is much greater in their natural environments than in environments that have been cultured, and these uncultured microbes belong to new families, classes, orders, and even kingdoms (Oren, 2002). Genome sequencing of hyperthermophile *Pyrobaculum aerophilum* was the first to be completed; genomes of *Aquifex aeolicus*, *Methanobacterium thermoautotrophicum*, *Methanococcus jannashii*, and *Archaeoglobus fulgidus* have been, or are nearly, completed.

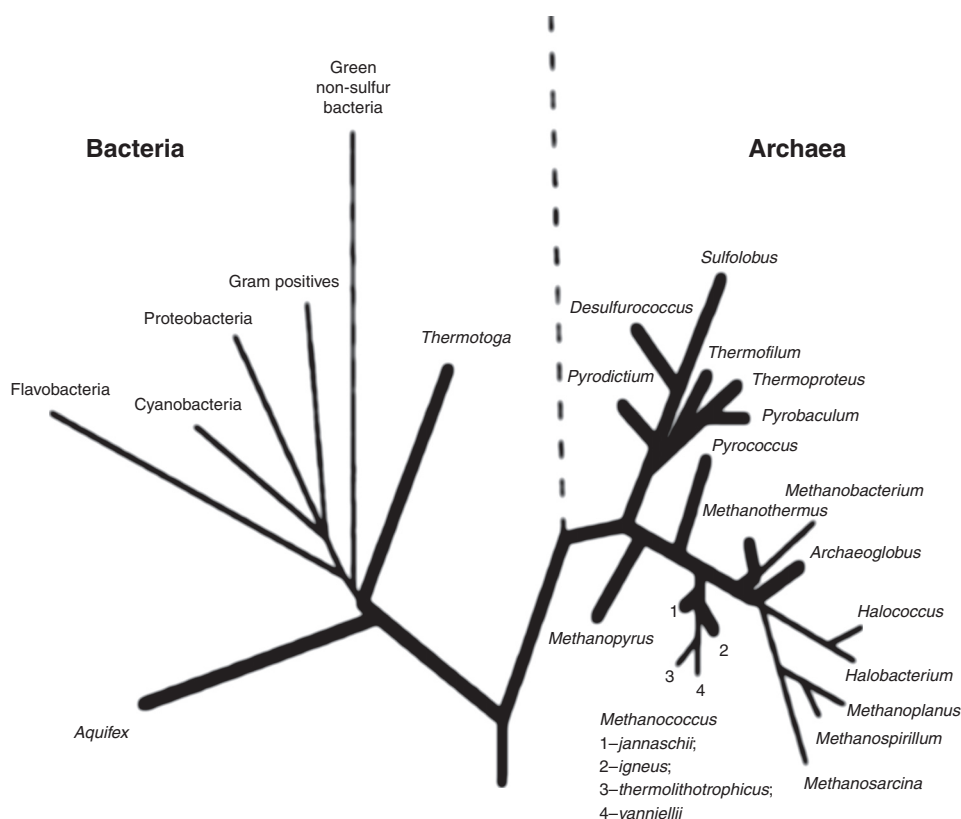


Figure 2.2. Hyperthermophiles (represented by bold lines) within the phylogenetic tree. [Modified from Stetter (1992) after Woese (1990). From Blöchl et al. (1995), with permission from Elsevier. Copyright © 1998.]

Survival Strategy. Thermophilic adaptation at elevated temperatures is the subject of contributions from many different physicochemical factors. A vast literature exists on the strategies adopted by thermophiles for their survival, as the research goes back to 1897 (England et al., 2003). Thermophiles are able to survive at high temperature, as they nurture enzymes and osmolytes in their womb. Thermophiles are robust at such high temperature because of the stability of their proteins and enzymes and the production of compatible solutes such as diinositol phosphate and diglycerol phosphate (Vesteinsdottir, 2008). Other key contributors to thermostability are the high GC content of their genome, the ratio of charged to uncharged amino acids, the increase in ionic interactions and hydrogen bonding, metal coordination and the compactness of their proteins, and the preference of certain amino acids (e.g., in *Bacillus stearothermophilus* it was reported that Gly is preferred over Ile and Ala over Tyr) (Trivedi, 2006). Posttranslational modification and chaperone-assisted folding of proteins are other noteworthy features of thermostabilization (Trivedi, 2006). England et al. (2003) reported that thermophilic adaptation in bacterial genomes happens by selection of more designable folds in their proteins' tertiary topology compared to that in their mesophilic counterparts.

Culturing Protocols. Isolation of thermophilic microbes took place using two methods: first, through standard microbial culture procedures, by which researchers discovered that heat-tolerant and spore-forming bacteria are the cause of many persistent contaminations, and second, through ecological studies of geothermal environments. Most thermophilic organisms have been isolated from continental geothermal and artificial thermal environments where the sodium level is low; few were isolated from marine hydrothermal environments (Santos et al., 1998). A major problem in culturing thermophiles in solid media using agar is that agar-based media exhibit syneresis at such high temperatures (Lin and Casida, 1984). An alternative medium—polysilicate (Huber et al., 2000) or Gelrite gellan gum (Kelco, United States), also known as PS-60 and S-60—was proposed as being superior to agar for the culture of thermophiles, due to their higher stability. It is a highly purified polysaccharide with the property of self-jelling. Moreover, there are relatively very few bacteria that demonstrate optimum growth at 100°C in pure culture.

Huber et al. (1995) developed an efficient and rapid method of isolation for hyperthermophiles from mixed cultures: a novel, plating-independent isolation procedure. The method, called the “optical tweezers trap,” is based on separation of a single cell from enrichment cultures by the use of a laser microscope and subsequent growth of this cell. They also devised a cell separation unit for the isolation of single cells. The cell can be separated by at least 6 cm from the mixed culture within 3 to 10 min. Kurtböke et al. (1993) used a bacteriophage for selective isolation of thermophilic actinomycetes. This turned out to be a novel method that involves exposing the test material to bacteriophage suspensions prior to inoculation on isolation plates. This method caused phage susceptibility of thermophilic bacteria, providing a selective means of reducing their numbers and increased the numbers of *Thermomonospora*, *Saccharopolyspora rectivirgula*, and thermophilic *Streptomyces* spp. on these media. Table 2.2 summaries of the important media recipes used to culture thermophiles.

To obtain the organism predicted using phylogenetic sampling, a new isolation strategy was developed by Huber and others (1995). This procedure combines an analysis of 16S rRNA gene sequences from the environment, specific whole-cell hybridization within enrichment cultures, and isolation of the morphologically identified single cell using the optical tweezers trap. For the first time, a novel hyperthermophilic archaeon was isolated, tracked by 16S rRNA analysis from a hot pond in Yellowstone National Park (the Obsidian Pool). In the future, this new strategy could also be applied to isolate uncultivated organisms from different biotopes, predicted phylogenetically by in situ 16S rRNA gene sequence analysis (Huber et al., 2000).

Limitations. Along with its immense industrial applicability, practical limitations coexist in culturing thermophiles. To state the major problems associated with culturing thermophiles under in vitro conditions, microbiologists often fail to identify the appropriate sample because of the availability of a vast number of samples (e.g., water, sediments, microbial mats) for isolating them (Akmar et al., 2011). A second problem lies in the use of solid agar-based media because agar exhibit syneresis at such high temperatures (Lin and Casida, 1984). Another problem is that relatively very few hyperthermophilic bacteria demonstrate optimum growth at such high temperatures in pure culture with low biomass yields.

Obstructions to obtaining thermophilic cultures on an industrial scale include strain instability, culture collapse, inappropriate substrate utilization, product inhibition,

TABLE 2.2. Isolated Thermophiles with Their Habitat and Media Formulations

Thermophile	Sample	Media	References
<i>Bacteria</i>			
<i>Bacillus</i> sp.	Soil gardens of Chancellor College, Malawi	Nutrient broth, Oxoid or Difco	Ward and Cockson, 1972
<i>Bacillus subtilis</i> , <i>Bacillus coagulans</i> , <i>Bacillus megaterium</i> , <i>Bacillus circulans</i> , <i>Bacillus brevis</i>	Broadleaf tobacco	Enrichment	English et al., 1967
<i>Bacillus</i> sp.	Soil	NH ₂ SO ₄ , 0.4%; K ₂ HPO ₄ , 0.2%; KH ₂ PO ₄ , 0.1%; MgSO ₄ ·7H ₂ O, 0.05%; and yeast extract, 0.1%	Tomita et al., 1999
<i>Clostridium thermohydrosulfuricum</i>	Water	Thermus agar (ATCC medium 697)	Elnasser et al., 2007
<i>Bacillus</i> sp.	Soil	Minimal media glucose, ammonium salt, phosphate buffer, and inorganic salts	Wiegel et al., 1979
<i>Bacillus</i> sp.	Manure pile	Minimal medium consisting of glucose, ammonium salt, phosphate buffer, and inorganic salts	Epstein and Grossowicz, 1969
<i>Mycobacterium phlei</i> GTIS10	Soil from coal processing or compost sites	Enrichment culture	Kayser et al., 2002
<i>Anaerobranca horikoshii</i>	Water and soil	M-5 medium	Engle et al., 1996
<i>Bacillus</i> sp.	Broadleaf Tobacco	Trypticase soy broth or tobacco infusion agar plates	English et al., 1967
<i>Desulfotomaculum solfataricum</i> sp. nov.	Solfataric mud pools	Bicarbonate-buffered medium	Goorissen et al., 2003
<i>Hydrogenobacter</i> sp.	Hot springs, Grensdalur, SW Iceland	DSM81 medium	Vesteinsdottir, 2008
<i>Geobacillus pallidus</i> , <i>Anoxybacillus flavithermus</i>	Hot springs in Jordan	Nutrient broth	Al-Batayneh et al., 2011
<i>Bacterium</i> ZW-1	Acid mine drainage sample, Dexing mine, China	Enrichment culture ^a	Wei-Min et al., 2009
<i>Fungi</i>			
<i>Chaetomium thermophile</i>	Pre heated wood chips	Malt agar, Emerson YpSs agar	Tansey and Brock, 1971
<i>Humicola lanuginosus</i>	Tobacco snuff	Emerson YpSs agar (Difco)	Tansey, 1975
<i>Thielavia albomyces</i>			
<i>M. pulchella</i> var. <i>sulfuria</i>			
<i>Talaromyces thermophilus</i>			
<i>Algae</i>			
<i>Synechococcus lividus</i> , <i>Mastigocladus laminosus</i>	River water and sediment from the Yellowstone Plateau	D medium or complex hot springs water medium	Jackson and Castenholz, 1975

^a (NH₄)₂SO₄, 3.0 g/L; Na₂SO₄, 2.1 g/L; MgSO₄·7H₂O, 0.5 g/L; K₂HPO₄, 0.05 g/L; KCl, 0.1 g/L and Ca(NO₃)₂·0.01 g/L; 30 g/L FeSO₄·7H₂O.

bioreactor wear, and inhibition by reactor material. Another problem associated with culturing aerobic and anaerobic thermophiles is the decreased solubility of biologically relevant gases at such high temperatures (Deming, 1986). Adams and Kelly (1998) reported that no genetic system has been developed for an organism that can grow at 100°C. The absence of mutants and classical molecular analyses is a limiting factor in increasing our fundamental knowledge of how life forms thrive at such temperatures (Adams and Kelly, 1998) and thus obtaining their pure isolates. Thus, future advances in culturing thermophiles would require new cultivation methods, the discovery of new thermostable gels, and pressurized bioreactors.

2.2.2. Psychrophiles

Milieu. Psychrophiles or cryophiles are organisms that survive in cold environments of 10 to 20°C (Riley et al., 2008). True psychrophiles are generally defined as organisms with optimum temperature below 15 to 20°C. Habitats of psychrophiles include alpine and arctic soils, high-latitude and deep ocean waters, polar ice, glaciers, snowfields, and cryoconite holes. Prominent geographical locations include the arctic, the antarctic, and large areas of permafrost (permanently frozen soil) in Siberia, Russia, Canada, and other northern countries. Microbes, some of which may be viable, have been found in ice cores drilled at Vostok Station at depths down to about 3600 m, close to the surface of the huge subglacial Lake Vostok (Price, 2000). Most isolated psychrophiles are from marine environments.

Biodiversity. Psychrophilic bacteria are represented in all three domains of life. Most psychrophiles are bacteria and archaea; a few are fungi and snow algae. Examples are *Arthrobacter* spp., *Psychrobacter* spp., and members of the genera *Halomonas*, *Pseudomonas*, *Hyphomonas*, and *Sphingomonas*. Many of these organisms are methanotrophs, sulfate reducers, iron oxidizers, and methanogens. One bacterium obtained by James T. Staley's group, *Polaromonas vacuolata*, is a classic example of a psychrophile with an optimal temperature for growth of 4°C (Madigan and Marrs, 1997), and temperatures above 12°C are too warm for its reproduction. Bowman et al. (1997b) isolated bacteria from the antarctic sea ice and classified psychrophilic bacteria in four phylogenetic groups: α - and γ -proteobacteria, the gram-positive branch, and the Flexibacter–Bacteroides–Cytophaga phylum.

Survival Strategy. Psychrophiles are able to cope with such low temperatures for their survival because they translate cold-evolved enzymes. The adaptation is achieved through increased flexibility in selected portions of their overall protein structure. Cold acclimatization of proteins is achieved through an increased number of Gly residues and a decreased number of Pro and Arg residues in loops, a low number of ion pairs, and increased hydrophobic interactions or hydrogen bonds compared with their mesophilic homologs (D'Amico et al., 2006). It is believed that nucleic-acid-binding proteins play a central role in the cold adaptation of psychrophiles. Five unique genes have been characterized from two cold-adapted archaea and are predicted to encode nucleic-acid-binding proteins (Feller and Gerdey, 2003). The presence of cold-shock proteins such as RNA chaperone CspA maintains both the cell cycle and the growth of psychrophiles (Berger et al., 1996). They also harbor certain antifreeze proteins of various sizes, which decrease the freezing point of cellular water, thereby inhibiting their growth (Feller and Gerdey, 2003).

Other than encoding, low-temperature stable protein psychrophiles adapt to temperature change by modifying their lipid bilayer to avoid gel-phase transition and drastic loss of membrane properties, which is achieved through reduction in the packing of acyl chains in the membrane (Feller and Gerdey, 2003).

Culturing Protocols. Success stories of the isolation of psychrophiles date back to 1908, when Tsiklinsky isolated a psychrophile from the excrement of a fish. In 1948, Brog isolated *Cytophaga psychrophila*, the first psychrophile to be well characterized (Morita, 1975). India has established a permanent research station, Maitri at Schirmacher Oasis, East Antarctica, and has launched a series of scientific expeditions since 1981, with many psychrophiles identified, including *Arthrobotrys ferox*, *Torulopsis psychrophila*, *Acremonium antarcticum*, and *Hormoconis resinae* (Singh et al., 2006). Willerslev used polymerase chain reaction (PCR) amplification of an 18S rRNA gene and identified a diversity of fungi, plants, algae, and protists extracted from 2000- and 4000-year-old ice core samples from northern Greenland (Willerslev, 1999; Price, 2000).

To isolate psychrophiles from their environmental niche, all sampling equipment used for collection and transfer must be kept cold and sterilized (Russel, 2006). Large samples of permafrost soils and ice cores are usually collected using motorized equipment, to avoid the risk of contamination associated with collecting small samples. Rogers et al. (2004) and Christner et al. (2005) developed and discussed a large number of protocols for the isolation of psychrophiles, which differs somewhat from that of other classes of microorganisms. They involve surface sterilization using detergents or other biocides, followed by removal of the outer layers before sampling the core center, which is thawed slowly in isotonic media (Russel, 2006). The best general medium for culturing marine psychrophiles was reported to be marine 2216 agar (Difco Laboratories). Dilute SWC and SWCm media of Irgens (1989) and R2A media (oxoid) are good for isolating fastidious psychrophiles (Paul, 2001). Alternatively, natural or artificial seawater with other media constituents added can be used. It is better to filter-sterilize such media rather than autoclaving. Media should be kept refrigerated before being used for isolation, purification, or subculturing. Culture plates are incubated either below 5°C or cryopreserved for a long incubation period. Most psychrophilic bacteria were reported to form visible colonies within one month at 5°C (Russel, 2006). General cryoprotectants used are glycerol or dimethyl sulfoxide. Media need to be supplemented with suitable antifungal agents such as cycloheximide and/or nystatin (Bowman, 2001) to prevent contamination during subculturing processes. Moreover, profuse aeration at low temperature by vigorous shaking of stationary cultures of psychrophiles produces higher cell crops indirectly by increasing the solubility and therefore the supply of O₂ (Sinclair and Stokes, 1964). Table 2.3 represents the media formulations of few isolated psychrophiles belonging to the three domains of life.

As fewer than 1% of the microbes present in our ecosystem can be cultivated based on 16S rRNA, real-time PCR, rrn operon, DNA microarray, or metagenomics, we can hope to isolate and identify fastidious psychrophiles in the near future. Recently sequenced whole genomes for psychrophiles *Colwellia psychrerythraea*, *Idiomarina loihiensis*, *Pseudalteromonas haloplanktis*, and *Psychromonas ingrahamii* (Riley et al., 2008) are such achievements.

Limitations. To date, no standard protocol or medium specific for isolation of psychrophiles has been made available, and collection of samples is from logistically difficult

TABLE 2.3. Habitat and Media Recipes of Some Isolated Psychrophiles

Species	Habitat/Source	Media	References
<i>Colwellia demingiae</i>	Sea ice, United States	Marine 2216 ^a	Bowman et al., 1988
<i>Colwellia hadaliensis</i>	Deep sea	Marine 2216	Denning et al., 1988
<i>Colwellia rossensis</i>	Sea ice	Marine 2216/SWCm ^b	Bowman et al., 1988
<i>Colwellia psycherythraea</i>	Sea ice, on surfaces of polar marine fauna, fish eggs	Marine 2216	Bowman et al., 1988
<i>Colwellia psychrotropica</i>	Saline meromictic lake water	Marine 2216	Bowman et al., 1988
<i>Glaciecola punicea</i>	Sea ice	Marine 2216	Bowman et al., 1988
<i>Glaciecola pallidula</i>	Sea ice	Marine 2216	Bowman et al., 1988
<i>Methylosphaera hansonii</i>	Saline meromictic lake	NMS-seawater	Bowman et al., 1997b
<i>Moritella japonica</i>	Deep sea (Japan Trench)	Marine 2216	Nogi et al., 1998
<i>Psychromonas antactica</i>	Ice-shelf pond sediment	Marine 2216	Mountford et al., 1998
<i>Octadecabacter antarcticus</i>	Sea ice	Marine 2216/SWCm	Gosink et al., 1997
<i>Octadecabacter arcticus</i>	Sea ice	Marine 2216	Gosink et al., 1997
<i>Desulvotalea psychrophila</i>	Marine sediment	<i>Desulfobacter postgatei</i> media (DSMZ medium 193)	Knoblauch et al., 1999
<i>Desulfofrigus oceanense</i>	Marine sediment (Arctic O)	DSMZ Medium 193	Knoblauch et al., 1999
<i>Desulfofrigus fragile</i>	Marine sediment (Arctic O)	DSMZ Medium 193	Knoblauch et al., 1999
<i>Desulfofaba gelida</i>	Sea ice, under ice sea water	DSMZ Medium 193	Knoblauch et al., 1999
<i>Gelidibacter algens</i>	Sea ice	Marine 2216	Bowman et al., 1997a
<i>Psychroflexus torquis</i>	Sea ice	Marine 2216	Bowman et al., 1998a
<i>Psychroserpens burtonensis</i>	Sea ice	Marine 2216 + 0.5% Tween 80	Bowman et al., 1997
<i>Polaribacter franzmannii</i>	Sea ice	SWCm	Gosink et al., 1998
<i>Polaribacter irgensii</i>	Sea ice	SWCm	Gosink et al., 1998
<i>Polaribacter filamentus</i>	Sea ice	SWCm	Gosink et al., 1998
<i>Methanogenium frigidum</i>	Saline lake sediment, water	Saturn holding medium	Franzmann et al., 1997
Bacteria, yeast	Mosses of tundra in Alaska deep-frozen fish dirt in a cold-room snow valley at the Taisetsu Mountains	Sabouraud's agar plate (agar containing peptone)	Saruyama et al., 1978

(continued)

TABLE 2.3. (Continued)

Species	Habitat/Source	Media	References
α - and γ -Proteobacteria	Juan de Fuca deep-sea hydrothermal area; samples were collected during <i>Atlantis</i> cruise in 2000 using the deep submersible vehicle <i>Alvin</i> China No. 1 glacier	Artificial sea water medium ^c	Edwards et al., 2003
<i>Flavobacterium xinjiangense</i> sp. nov. and <i>Flavobacterium omnivorum</i> sp. nov.		Peptone–yeast extract–glucose medium ^d	Zhu et al., 2003
<i>Leifsonia rubra</i> sp. nov. and <i>Leifsonia aurea</i> sp. nov.	Cyanobacterial mat sample from a pond in Wright Valley, McMurdo, Antarctica Soil from Abashiri (Hokkaido, Japan)	Antarctic bacterial medium Mineral synthetic medium	Reddy et al., 2003 Nakagawa et al., 2004
<i>Cryptococcus cylindricus</i> , <i>Mrakia frigid</i> , and <i>Cystoflobasidium capitatum</i> (yeast) <i>Clostridium</i>	Soil, mud, and sewage	Trypticase soy broth	Sinclair and Stokes et al., 1964
Bacteria	Soil samples from the manufacturing and assembly areas of the <i>Viking</i> spacecraft	Trypticase soy agar, Mycophil agar	Foster and Winans, 1975
Microfungi	Lichen and plants from Poland and Argentina <i>Soil taken from Livingston Island, South Shetland Archipelago, West Antarctica</i>	Malt yeast extract agar Cooke Rose Bengal agar	Moller and Dreyfuss, 1996 Kostadinova et al., 2009

Source: Adapted from Paul (2001).

^aMarine medium 2216 formula: peptone + yeast extract + ferric phosphate + artificial seawater (pH 7.5).

^bSWCm medium formula (Irgens et al., 1989): KH_2PO_4 + ferric citrate + NH_4Cl + yeast extract + beef extract + Hunter's mineral salts [$\text{nitroacetic acid} + \text{water and neutralized by adding KOH} + \text{MgSO}_4 + \text{CaCl}_2 \cdot 2\text{H}_2\text{O} + (\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{FeSO}_4 \cdot 7\text{H}_2\text{O}, \text{MnSO}_4 \cdot \text{H}_2\text{O}, \text{CuSO}_4 \cdot 5\text{H}_2\text{O}, \text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}, \text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}] + \text{C source (optional)} + \text{artificial seawater}$.

^cArtificial seawater formula (ZoBell, 1946): 0.002 g NH_4NO_3 , 0.027 g H_3BO_3 , 1.14 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.001 g $\text{Fe}(\text{PO}_4)_2$, 5.143 g MgCl_2 , 0.1 g KBr , 0.69 g KCl , 0.2 g NaHCO_3 , 24.32 g NaCl , 0.003 g NaF , 0.002 g $\text{Na}_2\text{O}_3 \cdot \text{Si} \cdot 9\text{H}_2\text{O}$, 4.06 g Na_2SO_4 , and 0.0263 $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$ dissolved in 1000 mL of distilled water. The chemicals can be added together dry and mixed thoroughly to make a large supply (add 35 g per liter of media).

^dPYG medium (L^{-1}): 5 g polypeptone, 5 g tryptone, 10 g yeast extract, 10 g glucose, and 40 mL salt solution. The salt solution, pH 7.2, contained (L^{-1}): 0.2 g CaCl_2 , 0.4 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g K_2HPO_4 , 1 g KH_2PO_4 , 10 g NaHCO_3 , and 2 g NaCl (Zhu et al., 2003).

sites requiring drilling with expensive equipment. The two main reasons why microbiologists in the past have not been successful in isolating psychrophilic bacteria were probably the improper selection of source material and the laboratory procedure for handling those materials. Source material selection is most crucial (Morita, 1975). The use of inappropriate media may fail to isolate those microorganisms growing actively in the psychrophilic niche, particularly if the salinity of the ecotype is ignored when the source sample is from a marine environment. Cold habitats often contain high concentrations of salts and other cryoprotective compounds. Hence the need is to add these requirements. The pour plate technique employing an agar medium cannot be recommended, as most marine forms are thermosensitive to the plating temperature of agar (Morita, 1975). Contamination by psychrotolerant microbes poses another problem in isolation of extreme psychrophiles. The microbes can be eliminated by maintaining the correct growth temperature for extreme psychrophiles with high-end equipment which shows little temperature variation.

2.2.3. Halophiles

Milieu. Halophiles are microorganisms that survive in high-saline environments requiring 0.5 M NaCl for optimal growth. The salinity of halophilic environments is almost 10 times that of normal ocean water. The major habitats of halophiles are salt lakes, brines, ponds, and soils. Salt deposits, some of them up to 1200 m in thickness, are found predominantly in the northern regions of the continents: in Siberia, Canada (Mackenzie basin), northern and central Europe (Zechstein series), southeastern Europe (Alps and Carpathian mountains), and the Midcontinent basin in North America (Zarkhov, 1981). The salt waters can be thalassohaline (e.g., the Great Salt Lake) or athalassohaline (e.g., the Dead Sea) hypersaline environments (Kunte et al., 2002), or a lake in which the concentration of divalent cations exceeds that of monovalent cations (Oren, 2002).

Biodiversity. The phylogenetic and metabolic diversity of microorganisms living at high salt concentrations is astonishing. Halophiles populate each of the three domains of life—Archaea, Bacteria, and Eukarya—and are also classified as oxygenic and anoxygenic phototrophs, aerobic heterotrophs, fermenters, denitrifiers, sulfate reducers, and methanogens (Oren, 2002). *Salinibacter ruber*, *Haloarchae*, *Halobacterium halobium*, and *Dunaliella salina* are a few prominent examples. Oren's research article included a figure (Fig. 2.3) that illustrates those branches of the phylogenetic tree of life where halophiles are able to grow at salt concentrations above 100 g/L. Most halophiles in the domain Bacteria are more moderate than extreme; however, a few resemble the archeal family Halobacteriaceae. It has been reported that the waters of the north arm of the Great Salt Lake have up to 2.4×10^8 cells/mL of extremely halophilic bacteria of the genera *Halobacterium* and *Halococcus*. Dombrowski (1963) isolated and reported *Bacillus circulans*, a halophile, inhabiting rock salt.

Survival Strategy. All halophiles are pleomorphic. The osmotic pressure of halophiles controls the amount of salt inside a cell. High levels of proline and glycerol in their cells maintain their osmolarity in a hypertonic environment: for example, for the production of the extremolyte ectoine, extraction of β -carotene from *Dunaliella*, production of polyunsaturated fatty acids from *Haloferax*, and bioremediation of contaminated

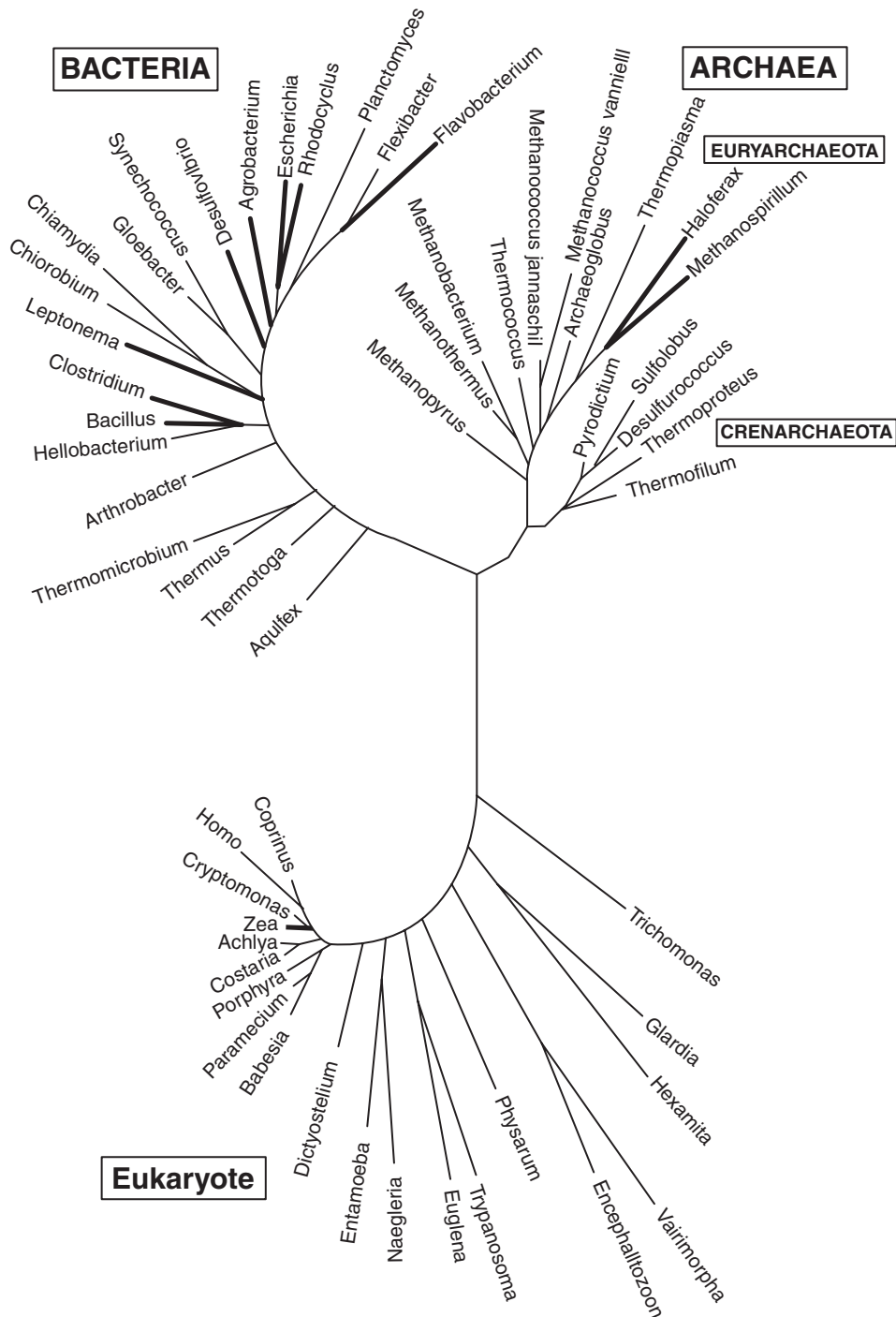


Figure 2.3. The universal phylogenetic tree of life based on small subunit rRNA gene sequences. Branches of halophilic microorganisms are shown in bold. [Adapted from Oren (2002), with permission from Springer Science+Media.]

hypersaline brine. Moreover, they produce alkali-stable DNAases, lipases, amylases, gelatinases, and proteases.

Culturing Protocols. The vast applicability of halophiles demands their isolation, culture, and characterization under in vitro conditions. Viable halophilicities have been isolated in England from salt deposits that originated in the Triassic and Permian periods (Denner et al., 1994). Extreme halophiles have been isolated from salt crystals from an English salt mine, Permian bedded salt in New Mexico, and rock salt from an Austrian salt mine deposit (Stan-Lotter, 1999). Techniques to isolate halophiles depend on the area from where they are to be obtained. Archeal halophiles are identified by red or pink pigmentation of the isolates, whereas extreme halophilic bacteria isolated from brine pools, brine injection fluids, and rock salt are white or yellowish in appearance (Stan-Lotter, 1999).

Halophiles grow over a wide growth range of salt concentration, and the requirement for NaCl above the physiological range is difficult to explain (Denner et al., 1994). Halophilicities dwelling in seawater are isolated using the membrane filter technique (Rodríguez, 1998), where samples are collected in containers disinfected with alcohol and filtrated through membrane filters which are then placed on petri plates with the required media (Table 2.4). Halophilicity is tested by the growth of individual microbial colonies in artificial seawater supplemented with 2 M NaCl. Pure cultures of those showing growth in such conditions are obtained by a repeated quadrant streak plate method. The media for culturing halophiles generally comprise 3 M NaCl, 50 to 100 ppm of potassium for normal pigmentation, and 0.1 to 0.5 M magnesium for correct morphology. The addition of 10 ppm of Fe^{2+} can increase cell production. The most rapid growth is obtained between 37 and 45°C. Table 2.4 lists various media for growing halophiles.

Robinson et al. (2005) have described a method of obtaining extreme halophiles in petri plates supplemented with halophilic media and penicillin. As the halophiles lack a peptidoglycan cell wall, they grow well under such conditions. Halophiles were isolated from rock salt by Denner et al. (1994) using samples collected after blasting from newly exposed of rocks faces, followed by storage in absolute ethanol. Sample pieces of about 2 g were dipped in ethanol, flamed, transferred to M2 medium (Tomlinson and Hochstein, 1972) and incubated at 37°C with shaking. When the culture became turbid, loopfuls were streaked in agar medium and purified further by repeated spreading on solid M2 medium (Denner et al., 1994). Mixing and aeration were maintained using a sparger at the bottom of the tank and an airflow of about 1 to 5 L/min. An inoculum of 5% yielded 5 to 6 g wet weight of cells per liter in 65 h.

Another consideration in the large-scale production of halophiles is the use of antifoaming agents such as Antifoam Compounds A and B (Braun MAZU DF 7960) (Kushner, 1966). Growth can also be obtained in large volumes using sparged air (Christian and Ingram, 1959). Due to the methods of enrichment, fast-growing halophiles are selected with the possibility of slow-growing haloarchaeal genera. Such slow-growing genera can be deduced from molecular analyses by PCR amplification of 16S rRNA genes and sequencing of cloned products. *Haloarcula* and *Halobacterium* species were detected on the basis of polar lipid composition (Norton, 1993), a technique that obviates culturing of microorganisms and has permitted the detection of novel and unexpected phylogenetic groups (Kunte et al., 2002). However, the complete genome sequence of the first halophile, *Halobacterium salinarum*, was published in 2002 (Oren, 2002). The composition of some complex media for growth of extreme halophiles is given in Table 2.5.

TABLE 2.4. Habitat and Media Recipes of Isolated Halophiles

No.	Organism	Habitat	Media	Type	References
1	F1-4	Sea water, Mediterranean coast between Alicante and Santa Pola	Eimhjellen or Sehgal and Gibbons media 2 M NaCl	Extreme halophile	Rodríguez-Valera et al., 1979
2	Strain SF1, DSM 3243	Solar salt pond A-22 of the Leslie Salt Co., San Francisco, CA	^a Enrichment medium containing trimethylamine (TMA)	Moderate methanogenic halophile	Mathrani et al., 1985
3	<i>Halobaculum gomorrense</i>	Dead Sea	^b Enrichment medium	Extreme halophile	Oren et al., 1995
	<i>Halorhabdus utahensis</i>	Great Salt Lake	^c Enrichment medium (EX-medium)	Extreme halophile	Wanio et al., 2000
4	<i>Halogeometricum borinquense</i>	Solar salterns, of Puerto Rico	^d Seghal–Gibbons medium glycerol solar salt medium	Extreme halophile	Montalvo-Rodríguez et al., 1998
5	<i>Halobacterium salinarum</i>	Thai fish sauce, salted fish and hides, solar salterns, estuaries polluted with crude oil, etc.	Microscope microdrill/micropipette system, fluids from brine inclusions were aseptically extracted from primary, hopper texture, Halite crystals from 8 and 85 m below the surface	Extreme halophile	Mormile et al., 2003
6	<i>Halococcus salifodinae</i>	Permian rock salt, Austria	M2 media (Tomlinson and Hochstein, 1972)	Extreme halophile	Denner et al., 1994
7	<i>Halothermothrix orenii</i>	Tunisian salt lake	^e Enrichment medium	Moderate halophilic thermophile	Cayol et al., 1994

^aIn g/L: NH₄Cl, 1.0; K₂HPO₄·3H₂O, 0.40; MgCl₂·6H₂O, 0.10; yeast extract (Difco), 0.50; diatomaceous earth, 100; Na₂S·9H₂O, 0.2; NaHCO₃, 2.0; TMA hydrochloride, 1.91 (20 mM). The gas phase was N₂, and the final pH was 7.3. For isolation enrichment medium, except diatomaceous earth was omitted and resazurin (1.0 mg/L) was added.

^bIn g/L: 125 g of NaCl, 160 g of MgCl₂·6H₂O, 5.0 g of K₂SO₄, 1.0 g of CaCl₂·2H₂O, 1.0 g of yeast extract (Difco), 1.0 g of Casamino acids (Difco), and 2.0 g of soluble starch (BDH).

^cIn g/L: NaCl, 220; NaBr, 0.1; MgSO₄·7H₂O, 20; KCl, 5; NH₄Cl, 2; NaHCO₃, 0.2; KH₂PO₄, 0.5; yeast extract (Difco), 2; trypticase peptone (BBL), 1; and trace-metal solution (TMS 3), 2 mL (Ingvorsen and Jørgensen, 1984). CaCl₂ solution (CaCl₂·6H₂O, 100 g/L) and 2 mL sterile FeCl₂, MnCl₂ solution (FeCl₂·4H₂O, 20 g/L MnCl₂·4H₂O, 20 g/L).

^dIn g/L: 250 solar salt, 10 glycerol, and 1 Casamino acids.

^eIn g/L: NH₄Cl, 1.0; KH₂PO₄, 0.3; K₂HPO₄, 0.3; MgCl₂·6H₂O, 2.0; CaCl₂·2H₂O, 0.2; KCl, 4.0; CH₃COONa·3H₂O, 1.0; glucose, 10.0; NaCl, 100; bio-tryptase (bioMkriex), 3.0; yeast extract (Difco), 3.0; resazurin (0.1% w/v), 1 mL; trace element solution (7), 1 mL.

TABLE 2.5. Composition of Complex Media Used for Growing Extreme Halophiles (g/100 mL)

Components	Weber, 1949	Katznelson and Lochhead, 1952	Brown and Gibbons, 1955	Abram and Gibbons, 1961	Sehgal and Gibbons, 1960	Dundas et al., 1963	Eimhjellen, 1965
Casamino acids VF	—	1.5	—	—	—	—	—
Casamino acids ^a	—	—	1.5	0.5	0.75	—	—
Yeast extract ^a	—	—	—	1.0	1.0	1.0	0.5
Proteose peptone ^a	—	—	—	0.5	—	—	—
Tryptone ^b	—	—	—	—	—	—	—
Gelatin hydrolysate	0.1	—	—	—	—	—	—
Arginine	0.1	—	—	—	—	—	—
Cysteine	—	0.01	—	—	—	—	—
Tryptophane	—	0.01	—	—	—	—	—
Na citrate	0.06	0.05	0.3	0.3	0.3	—	—
Na succinate	0.2	0.2	—	—	—	—	—
NaH glutamate	0.3	—	—	—	—	—	—
Glutamic acid	—	—	0.25	—	—	—	—
K ₂ HPO ₄	0.1	0.05	—	—	—	—	—
KH ₂ PO ₄	—	0.05	—	—	—	—	—
KNO ₃	—	0.01	—	—	—	—	—
KCl	—	—	0.2	0.2	0.2	0.5	—
MgSO ₄ ·7H ₂ O	5.0	2.0	2.5	2.0	2.0	—	2.0
MgCl ₂ ·6H ₂ O	—	—	—	—	—	0.5	—
NaNO ₃	2.0	—	—	—	—	—	—
FeSO ₄ ·7H ₂ O	0.002	0.0005	—	0.005	0.0023	—	—
FeCl ₂	—	—	—	—	—	—	—
NH ₄ Cl	—	—	—	—	—	0.5	—
CaCl ₂ ·2H ₂ O	—	—	—	—	—	—	0.5
NaCl	22.0	22–26	22	20–30	25	25	25 ^c
Other salts	—	—	—	—	—	—	—
pH final adjustment	6.5–6.8	6.5–6.8	7.0	7.2–7.4	7.4	—	—

^aDifco.

^bOxoid.

^cSolar salt.

Source: Adapted from N. E. Gibbons, *Methods in Microbiology*, Vol. 3, Pt. B, with permission from Elsevier. Copyright © 1969.

Limitations. Success stories relating to the isolation and culture of halophiles exist because hypersaline habitats are common throughout the world, but extremely hypersaline habitats are rather rare and difficult to explore, as they are always in hot, dry areas. Additionally, culturing halophiles is costly because of the added expense involved in the use of glass and plastic apparatus. Moreover, these cultures generate an offensive odor and thus the apparatus must be installed under a fume hood. An adequate oxygen supply is necessary for the growth of halobacteria, but a constraint is the solubility of oxygen in a high salt concentration.

When different strains of halobacteria are used in a laboratory, cross-contamination is a notable problem. Certain genera of halophiles, such as *Halofemx mediterranei* and *Haioarcula hispanica*, produce exopolymers that adhere to surfaces of culture vessels or spargers in forced-air systems. A sparger must be cleaned at intervals, as it becomes plugged in continuous culture systems. A related problem with liquid cultures of halobacteria is foam formation. Concentrated salts tend to favor foam formation, and the problem is worsened if the strain is an exopolymer producer. Serious difficulties have occurred in controlling foam formation in high-biomass H11 *mediterranei* cultures in fermentors even when using very effective antifoam compounds (Rodríguez-Valera et al., 1995.). In large-scale production of halophiles, although maintaining proper media and aeration conditions leads to culture scaleup, there is still much to be learned about the growth patterns and requirements of these microorganisms (Hungate, 1969). Moreover, due to the methods of enrichment, fast-growing halophiles are selected. Slow-growing haloarchaeal genera cannot be detected using general microbial technologies in culturing them.

2.2.4. Alkaliphiles

Milieu. Microorganisms inhabiting extreme alkaline environments are termed alkaliphiles (Ulukanli, 2002). They consist of two main physiological groups: alkaliphiles and haloalkaliphiles (Horikoshi, 1999). Alkaliphiles have important industrial applications in the production of proteases and lipases used in biological detergents. Alkaliphiles live in soils laden with carbonate and in soda lakes, such as those found in Egypt, the Rift Valley of Africa, and the western United States (Madigan et al., 1997). Naturally occurring stable alkaline environments are of two kinds: high- and low- Ca^{2+} environments. These include locations in Cyprus, California, Yugoslavia, Oman, Turkey, and Jordan (Seckbach, 1999), and the alkaline Lonar Lake in India, formed by meteorite impact, is another unique location (Kanekar et al., 2008). Highly alkaline (pH 11 to 12) aerobic and anoxygenic phototrophic bacteria, mainly of the genera *Ectothiorhodospira* and *Halorhodospira*, can be found in the Rift Valley (Grant and Horikoshi, 1992). Alkaliphilic bacteria have also been isolated from deep-sea sediments collected from depths up to 10,898 m in the Mariana Trench (Horikoshi, 1999). In addition to the use of lipases, pullulanase, and proteases as detergent additives, alkaliphilic pectinases are utilized for quality improvement of paper by making it stronger and nonwoody (Horikoshi, 1999).

Biodiversity. Alkaliphiles have existed since archaean times and include prokaryotes, eukaryotes, and archaea (Horikoshi, 1999). Common examples are *Natronococcus*, *Geoalkalibacter ferrihydriticus*, *Bacillus okhensis*, *Alkalibacterium iburiense*, and *Spirulina maxima*. Other alkaliphilic bacteria are members of the Actinomycetes, Bacillaceae, Clostridiaceae, Hatonnaerobiales, and γ -Proteobacteria (Schaechter and Lederberg, 2004).

Survival Strategy. Alkaliphiles face many challenges in an alkaline environment. They require maintaining intracellular pH near neutrality by constantly pumping protons into their cytoplasm (Horikoshi, 1999). Speelmans et al. (1993) reported that *Bacillus subtilis* and *Vibrio alginolyticus* have evolved mechanisms for acidification of cytoplasm relative to the external pH. The cell membrane also plays a key role in the survival strategies of alkaliphiles. The cell membrane comprises certain acidic polymers, such as galacturonic acid, gluconic acid, glutamic acid, aspartic acid, teichuronic acid, and phosphoric acid, in addition to the usual phospholipids functioning as a negatively charged matrix to reduce the pH at the cell surface (Aono and Horikoshi, 1983). Work in extremely alkaliphilic *Bacillus* spp. showed that their plasma membrane maintains pH homeostasis by Na^+/K^+ antiporters (Hamamoto et al., 1994). Internal pH homeostasis is also achieved by passive regulation of cytoplasmic pools of polyamines and low membrane permeability (Bordenstein, 2008).

Culturing Protocols. Isolation of alkaliphiles dates back to 1934, when Vedder isolated the obligate alkalophile *Bacillus alkalophilus*. Alkaliphiles have been isolated mainly from neutral environments with a frequency of 10^2 to 10^5 g^{-1} of soil and haloalkaliphiles from extremely saline marine environments (Horikoshi, 1991, 1999). They have also been isolated from the gut of termites of the family Termitinae, which has pH 10 and is K^+ -rich (Thongram et al., 2003). The media most utilized for the isolation of alkaliphiles are Horikoshi I (Horikoshi, 1999), Horikoshi II (Horikoshi, 1999), nutrient agar of pH 10 with 30 g/L sodium chloride, Davis Mingiolis synthetic medium with pH 10 and 5 g/L peptone (Cruickshank et al., 1965), Tindall's medium (Tindall, 1988), and Lonar Lake water medium (Kanekar et al., 2008).

Details of habitat and media recipes that aid in the isolation of alkaliphiles are presented in Table 2.6. The optimum growth rate of alkaliphiles is obtained at a pH value at least two units above neutrality. The pH of small-scale cultures is controlled by Na_2CO_3 or $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ (pH 10 to 11), Borax/NaOH, and $\text{Na}_2\text{HPO}_4/\text{NaOH}$ buffer systems (pH 9 to 12). In large-scale bioreactors pH is maintained over 11 by NaOH and pH control systems (Grant and Tindall, 1980). One advantage in culturing alkaliphiles is that steps for media sterilization can be omitted, as high pH renders media less prone to contamination. A large number of alkaliphilic *Bacillus* spp. have been isolated in the past by Horikoshi and co-workers (Horikoshi, 1971). A few archeal species have also been isolated. Alkaliphiles can be maintained under in vitro conditions by routine subculturing, cryopreservation under liquid nitrogen, and as glycerol stocks at -80°C . The latter approach is widely practiced in laboratories that have limited equipment.

Limitations. One problem associated with isolation of alkaliphiles is that environmental conditions in soil systems differ widely from laboratory in vitro conditions. While maintaining small-scale cultures under laboratory conditions, buffering systems of Na_2CO_3 or $\text{Na}_2\text{CO}_3 \cdot 10\text{H}_2\text{O}$ are affected by atmospheric CO_2 . Preparation of solid agar media, Na_2CO_3 , needs to be autoclaved separately from the agar to ensure its proper solidification and to avoid darkening. Preparing media without Na_2CO_3 leads to lowering of pH to the acidic range of the carbonate-free media before autoclaving affects the agar adversely. The ability of alkaliphiles to maintain different pH profiles by utilizing different growth substrates makes their characterization difficult under chemostatic conditions in which a constant pH value is maintained.

TABLE 2.6. Habitat and Media Recipes of Some Isolated Alkaliphiles

Organism	Habitat	Media	References
<i>Bacillus flexus</i> XJU-3	Sampled from alkaline sewage in Shihezi City, Xinjiang	LB medium	Zhao et al., 2008
<i>Bacillus flexus</i> spp. NJY2 and NJY4	Maize-processing wastewater	Nejayote medium	Sanchez-Gonzalez et al., 2011
<i>Mycobacterium</i> sp. strain MHP-1	Soil sample collected at Chiba Prefecture	^a Carbon-free minimal medium	Habe, 2004
<i>Amphibacillus</i> sp. KSUCr3	Sediment, soil, and water sample from hypersaline soda lakes, Wadi Natrun valley in northern Egypt	^b Alkaline agar medium (pH 10.5)	Ibrahim et al., 2011
<i>Bacillus flexus</i>	Mangrove soil. Kakinada district, AP, India	Poly-peptone yeast glucose medium at pH 10.5	Kannan et al., 2009
<i>Bacillus</i> sp.	Alkaline soil (soil sample from a chicken run)	Alkaline <i>Bacillus</i> medium	Vallian et al., 2005
<i>Alkaliphilus metalliredigens</i> sp. nov.	Leachate ponds at U.S. Borax Company Boron, CA.	K ₂ HPO ₄ , 5.7 mM; (NH ₄) ₂ SO ₄ , 12.5 mM; NaCl, 327 mM; Na ₂ CO ₃ , 19.8 mM; Na ₂ B ₄ O ₇ , 10 mM; yeast extract, 0.025 g/L, 100 × mineral solution, 10 mL; the pH was adjusted to approximately 9.5	Ye et al., 2004
<i>Clostridium paradoxum</i> sp.	Sewage plants in the United States	Yeast extract–tryptone medium	Li et al., 1993
<i>Salinicoccus alkaliphilus</i> sp. nov.	Baer Soda Lake in Mongolia, Autonomous Region, China	Glucose 10 g/L; polypeptone 5 g/L; yeast extract 5 g/L; K ₂ HPO ₄ 1 g/L; MgSO ₄ ·7H ₂ O 0.2 g/L; NaCl, 100 g/L; Na ₂ CO ₃ , 10 g/L	Zhang et al., 2002
<i>Natrialba hulunbeirensis</i> sp. nov., <i>Natrialba chahamaoensis</i> sp. nov.	Soda lakes in Inner Mongolia Autonomous Region, China	Growth media had 7.5 g/L Casamino acids (Difco), 10 g/L yeast extract (Difco), 3.0 g/L trisodium citrate, 0.3 g/L MgSO ₄ ·7H ₂ O, 2.0 g/L KCl, traces of Fe ²⁺ and Mn ²⁺ , 200 g/L NaCl, and 8.0 g/L Na ₂ CO ₃ ; cultures were maintained in medium of Payne et al., 1960	Xue, 2001
<i>Paenibacillus</i> sp. SM-XY60	Gut of termites, Thailand	Alkaline K ⁺ medium	Thongaram et al., 2003

Source: Adapted from H. Santos, P. Lamosa, and N. Borges, *Methods in Microbiology*, Vol. 35, with permission from Elsevier. Copyright © 2006.

^a2.2 g Na₂HPO₄, 0.3 g KH₂PO₄, 3.0 g NH₄NO₃, 0.2 g MgSO₄·7H₂O, 10 mg FeSO₄·7H₂O and 10 mg CaCl₂·2H₂O per liter; pH 9–10 with appropriate amounts of Na₂CO₃, NaHCO₃, and/or NaOH; pyrene as sole C source.

^bGlucose (10 g/L), yeast extract (5 g/L), casamino acids (5 g/L), peptone (5 g/L), NaCl (100 g/L), Na₂CO₃ (15 g/L), agar (15 g/L), 300 μL of trace element solution, and K₂CrO₇ (1–20 mM). The trace element solution contained CaCl₂·2H₂O (1.7 g/L), FeSO₄·7H₂O (1.3 g/L), MnCl₂·4H₂O (15.4 g/L), ZnSO₄·7H₂O (0.25 g/L), H₃BO₃ (2.5 g/L), CuSO₄·5H₂O (0.125 g/L), Na₂MoO₄ (0.125 g/L), CoNO₃·6H₂O (0.23 g/L), and 2.5 mL of 95–97% H₂SO₄.

2.2.5. Acidophiles

Milieu. Acidophiles thrive under highly acidic conditions, preferably at pH 2.0 or below. Acidic environments are often associated with volcanic activity. Low-pH environments can also be caused by microbial activity and by chemoautotrophic oxidation of sulfide, elemental sulfur, and other reduced sulfur compounds, leading to formation of sulfuric acid, which acidifies the environment or medium (Ehrlich, 1963; Harrison, 1984). Extremely acidic environments (pH < 3) occur in some geothermal and coastal areas, most associated with anthropogenic activities such as the mining of coal and metal ores (Johnson et al., 1992). Interestingly, *Acidithiobacillus* and *Leptospirillum*, *Thermoplasmales* archaea, are also present in syntrophic relationships in concrete sewer pipes (Vincke et al., 2001; Brazelton et al., 2006). The Tinto River in Spain is a well-known hot spot for acidophiles.

Biodiversity. Acidophiles populate the three domains of life. A few prominent examples are the orders Sulfolobales (Crenarchaeota) and Thermoplasmatales (Euryarchaeota), the phylum Acidobacterium (Bacteria), *Acontium cylatium* (fungi), *Cyanidium caldarium* (red alga), and *Dunaliella acidophila* (green alga) (<http://serc.carleton.edu/microbelife/extreme/acidic>). The most acidophilic bacterium known is *Picrophilus oshimae*, with an optimal pH for growth of 0.7. Acidophilic organisms find use in mining for extracting trace metals through bioleaching. In the field of research, *Acidithiobacillus ferrooxidans* is the model organism among acidophiles, as its entire genome is sequenced (Hallberg, 2010).

Survival Strategy. Acidophiles cannot only tolerate, but can thrive in a very low pH environment. This is achieved mainly by maintaining a circumneutral intracellular pH (Baker-Austin and Dopson, 2007) and by membrane impermeability to protons to maintain a neutral cytoplasmic pH, as observed in *A. ferrooxidans* (Apel et al., 1980). They are also capable of extruding protons against the pH gradient with unique transport proteins. Tetraether lipids in the membranes of acidophiles such as *Thermoplasma acidophilum* renders them highly impermeable to protons (Baker-Austin and Dopson, 2007). Ether linkages characteristic of acidophilic membranes are less prone than ester linkages to acid hydrolysis (Golyshina and Timmis, 2005). Tyson et al. (2004) reported a variety of genes involved in cell membrane biosynthesis, indicative of a complex structure in microorganisms' acid-tolerance capacity. Selective reduction in the size of membrane channel proteins such as in *A. ferrooxidans* porin protein can render them acid tolerant (Amaro et al., 1991).

A further mechanism employed for acid tolerance involves reduction of proton influx by a chemiosmotic gradient (inside positive by influx of K⁺ ions) created through the Donnan potential by positively charged molecules (Baker-Austin and Dopson, 2007). Moreover, acidophiles have proton efflux protein systems to pump excess protons from their cytoplasm (Tyson et al., 2004). The presence of cytoplasmic buffer molecules capable of sequestering protons maintains pH homeostasis (Baker-Austin and Dopson, 2007). It was also observed that heterotrophic acidophiles degrade organic acid (act as uncouplers at the low pH of the respiratory chain, leading to dissociation of protons) for acid tolerance (Ciaramella et al., 2005). In this context, acidophiles such as *Picrophilus torridus* were reported to possess genes for an organic acid degradation pathway (Angelov and Liebl, 2006). In extreme acidophilic genomes, functional characterization of a large number of DNA and protein

repair genes such as chaperones provides a clue as to their mechanism of acid homeostasis (Baker-Austin and Dopson, 2007). The genomes of acidophiles also contain a large number of pyrimidine codons (less susceptible to acid hydrolysis) for protection from acid stress (Baker-Austin and Dopson, 2007). The presence of a high proportion of iron proteins can also contribute to acidic pH stability (Baker-Austin and Dopson, 2007).

Culturing Protocols. Archaea, bacteria, fungi, algae, and protozoans have been isolated from, and shown to be active in, extremely acidic sites (Johnson et al., 1992). Except for some heterotrophic species of the bacterial genus *Bacillus*, all organisms isolated from acidic environments with temperatures above 50°C have been archaea of the orders Sulfolobales and Thermoplasmatales (Schleper et al., 1995). Waksman and Joffe were the first to isolate the acidophile *Acidithiobacillus thiooxidans*, in 1929 (Schaechter, 2009). *Thiobacillus ferrooxidans* isolated some years later from acid mine drainage became the best characterized acidophile (Schaechter, 2009). Most researchers focus on the isolation of iron-oxidizing acidophiles since their metal mobilizing activities are exploited in the biological processing of sulfidic ores, and also because their oxidation of mineral sulfides is a primary cause of acid mine drainage pollution (Johnson et al., 1992). Several strains of acidophilic iron- and sulfur-oxidizing bacteria have been isolated from a long-abandoned copper mine in Norway (Johnson et al., 2001). Culturing acidophiles in liquid media is not problematic, but solid media pose a problem (Johnson, 1995). In solid medium some inhibitory material can complex with the gelling agent and result in nutrient deficiency. The choice of solid media for isolating acidophiles depends on the nature of the habitat of the isolate (e.g., field samples or defined laboratory cultures) and on the acidophilic bacteria that are being targeted (Johnson, 1995). Successful solid media for culturing acidophilic bacteria are listed in Table 2.7.

FeTSB is a dual-purpose medium that contains ferrous iron and organic substrates in the tryptone soya broth (TSB) component. FeTSBo, an overlay variant of FeTSB, has a high plating efficiency for over 50 strains of iron-oxidizing acidophiles. FeSo is the mixed medium most favored for culturing acidophiles containing potassium tetrathionate, in addition to iron and TSB. Washed agarose/yeast extract (WAYE) medium was designed to facilitate the growth of oligotrophic and heterotrophic acidophiles from various environmental samples (Johnson, 1995). FeTSBo, FeSo, and WAYE can be used to culture autotrophic, heterotrophic, and mixotrophic iron- and sulfur-oxidizing acidophiles from acid mine drainage and other environmental samples. YE (yeast extract) medium can be used to isolate heterotrophic acidophiles. FeTSBo and FeSo can be used to culture moderately thermophilic acidophiles (Johnson, 1995).

A notable problem associated with culturing acidophiles is in the use of agar for solid media, as agar is prone to hydrolysis under acidic conditions. Thus, it is necessary to sterilize the agar separately and to combine it with the other acidic media components when the solution is still warm. Moreover, agar-gelled acidic media are prone to hydrolysis of the polysaccharide, resulting in the accumulation of soluble oligo- and monosaccharides within the gel. An alternative to agar is Gelrite, with its high plating efficiencies. Agarose, a derivative of agar, is a more favored alternative gelling agent for solid media designed for acidophiles (Johnson, 1995).

The favorite method for culturing heterotrophic acidophiles is in a double-layer overlay solid medium in which *Acidiphilium* SJH is present in the underlayer gel. Colonies of acidophiles were reported to be larger and more gelatinous on overlaid media than WAYE

TABLE 2.7. Habitat and Media Recipes of Some Isolated Acidophiles

Organism	Habitat	Media	References
<i>Bacteria</i>			
<i>Picrophilus oshimae</i>	Solfatara in Japan	E. A. Freundt media	Schlepper et al., 1995
<i>Picrophilus torridus</i>	Disused pyrite mine, Conwy Valley, North Wales	FeTSB medium	Johnson et al., 1992
CCH7 bacteria	Abandoned subarctic copper mine in central Norway	FeTSB medium	Johnson et al., 2001
<i>Acidithiobacillus ferrooxidans</i>	Acid mine drainage treatment plant	9K medium and modified washed agarose/yeast extract medium with ferrous sulfate	Joe et al., 2007
HIB4 bacteria	Acid mine drainage, Gaofeng Mine, China	9K enrichment medium purified on solid ferrous-agarose medium	Yang et al., 2008
<i>Acidithiobacillus ferrooxidans</i>	Acid mine drainage samples, Dexing mine, Jiangxi Province, China	^a Enrichment medium	Wei-Min et al., 2009
Bacterium ZW-1	Acid mine drainage of Gaofeng Mine, Guangxi Province, China	9K enrichment medium purified on solid ferrous-agarose medium	Yang et al., 2008
Strain GF	Mine	Heterotrophic basal salts supplemented with a trace element mix with ferrous iron (at 10–25 mM) as electron donor	Hallberg, 2010
<i>Acidithiobacillus ferrivorans</i> sp. nov.			
<i>Fungi</i>			
<i>Teratosphaeria acidotherma</i>	Sainokawara hot spring, Japan	PDA-G plates ^b YM liquid medium	Yamazaki et al., 2010

^aIn g/L: (NH₄)₂SO₄, 3.0; Na₂SO₄, 2.1; MgSO₄·7H₂O, 0.5; K₂HPO₄; 0.05; KCl, 0.1; Ca(NO₃)₂, 0.01; FeSO₄·7H₂O, 30.

^b0.3% yeast extract (Difco), 0.3% malt extract (Difco), 0.5% peptone (Difco), 1% glucose (Wako), and 100 ppm tetracycline hydrochloride, pH 1.0.

or than nonoverlaid FeTSB or yeast extract medium gelled with acid-washed agarose (Johnson, 1995). Another approach to culturing acidophiles was reported by DeBruyn et al. in 1990 using polycarbonate filters floating on acidic, ferrous sulfate liquid medium, as the isolate failed to grow on any of the solid media tested. Overlaid FeTSB medium can be adapted (as FeSo) to promote the growth of sulfur-oxidizing acidophiles such as *Thiobacillus thiooxidans* while facilitating the growth of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and allowing colonies of iron oxidizers to be readily differentiated. Alternatively, by lowering the pH of FeTSBo medium, it is possible to produce colonies of iron-oxidizing acidophiles that are free of ferric iron precipitates. A less laborious way than plating methods to identify acidophiles and isolate them from the environment is through analysis of genomic diversity, such as GC content, DNA–DNA hybridization, rRNA analysis, and PCR, such as randomly amplified polymorphic DNA analysis of the 16S–23S intergenic spacer region (Pizzaro et al., 1996). Several acidophilic strains were detected from mining regions using a PCR-based approach (Wulf-Durand et al., 1997). A metagenomics approach is feasible, as the *Thermoplasma acidophilum* DSM 1728 genome was sequenced and released in September 2000 by the Max-Planck Institute (Ruepp et al., 2000).

Limitations. A large number of acidophiles still await isolation, due to the limitations, associated with culturing them. Speaking more specifically, culturing acidophiles in liquid media is not problematic; however, solid media pose a problem (Johnson, 1995). In solid media some inhibitory material can complex with the gelling agent and result in nutrient deficiency. A notable problem associated with culturing acidophiles is in the use of agar for solid media, as it is prone to hydrolysis under acidic conditions. Thus, it is necessary to sterilize the agar separately and to combine it with the other acidic media components when the solution is still warm. Moreover, agar-gelled acidic media are prone to hydrolysis of the polysaccharide, resulting in the accumulation of soluble oligo- and monosaccharides within the gel. Moreover, culture methods used routinely often fail to select slow-growing acidophiles, which are diluted due to competition from acidophiles such as *Acidithiobacillus ferrooxidans*, which tend to grow more rapidly and outgrow species such as *Leptospirillum* (Schaechter, 2009). A road block in the large-scale production of acidophiles is that complex substrates cannot be utilized for large-scale production because the growth of many acidophilic heterotrophs is inhibited by high concentrations of organic substrates in solid or liquid complex media (Johnson, 1995).

2.2.6. Barophiles

Milieu. Barophiles or psiozophiles are microorganisms that inhabit regions of high atmospheric pressure, displaying optimal growth at pressures above 40 MPa. Deep-sea and hydrothermal vents satisfy such requirements. The hydrostatic pressure in the sea increases by 1 atm (0.1 MPa) every 10 m. The mean depth of the oceans is about 4 km, equivalent to a pressure of 400 atm or 40 MPa, and the deepest parts of the ocean are more than 10 km deep. In these habitats, barophilic psychrophiles occupy areas below 2000 m (Yayanos, 1986), the upper layers being inhabited by barotolerant species (Jannasch and Wirsén, 1984). Obligate barophiles have been isolated from a depth of 6350 m (Yayanos, 1986). Barophilic hyperthermophiles can be isolated from hydrothermal vents located at a depth of at least 2000 m.

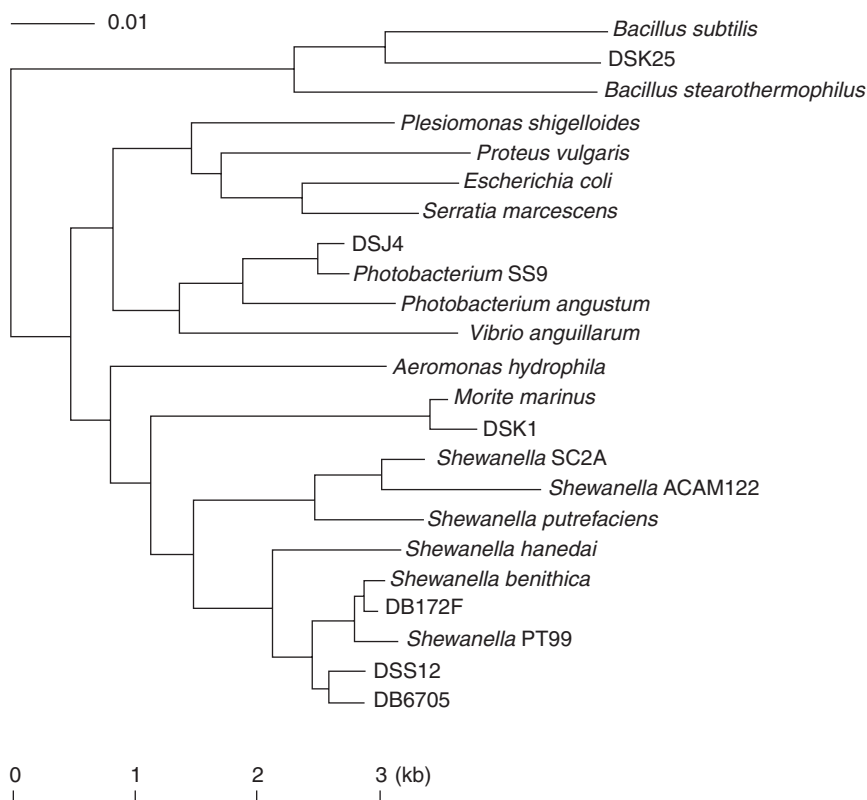


Figure 2.4. Phylogenetic trees showing the relationships between isolated sequences using the neighbor-joining method (Sailou and Nei, 1987). Deep-sea-adapted bacteria within the γ -subgroup of the Proteobacteria. The scale represents the average number of nucleotide substitutions and the genus *Bacillus* as determined by comparing 16S ribosomal DNA per site. [Adapted from Kato and Bartlett (1997), with permission from Springer Science+Media.]

Biodiversity. Most isolated barophiles are heterotrophic gram-negative bacteria and the preeminent genera of cultivated barophiles include *Shewanella*, *Photobacterium*, *Colwellia*, and *Moritella* (Wirsén et al., 1987). A 16S rRNA sequence of various barophiles showed that they belong to γ -proteobacteria (Kato, 1996) (Fig. 2.4). The majority of them belong to the genus *Shewanella*, *Thermococcus profundus*, and *Pyrococcus horikoshii* isolated deep-sea hydrothermal vents in the western Pacific Ocean are other good examples of barophiles. In 2005 the entire genome of a moderate barophile, *Shewanella violacea*, which grows optimally at 30 MPa and 8°C, was sequenced (Nakasone, 2005) and is treated as a model organism for barophily.

Survival Strategy. Barophiles adapt to tolerance of high pressure by homeoviscous adaptation, resulting in tight packing of their lipid membranes and increase in the level of unsaturated fatty acids (Lauro and Bartlett, 2007). Yano et al. (1998) reported that barophilic isolates maintain proper membrane fluidity because they contain polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA), which have a low lipid melting

point. Survival at high pressure also requires robust DNA repair systems (Rothschild and Mancinelli, 2001). Researchers have also found highly conserved pressure-regulated operons in barophiles, which help in their survival under high pressure (Kato, 1995, 1996). It has also been reported that transcriptional efficiency of various ribosomal proteins is responsible for high-pressure adaptations (Nakasone, 2005). Lauro and Bartlett (2007) reported that in barophiles elongated helices occur in the 16S rRNA genes and their frequency increases with increase in pressure. These helix changes are correlated with an improvement in ribosome function under high-pressure conditions. Research work by Giulio in 2005 provided some important insights into the role of amino acids in rendering proteins stable at high pressure and concluded that polar and small amino acids contribute more to barophilicity. These two amino acid properties are thought to be important for the origin of a universal genetic code and support the hypothesis that genetic code structuring took place under high hydrostatic pressure. The presence of proteins related to the heat-shock proteins in barophiles such as *Thermus barophilus* also aid in survival at elevated pressures (Marteinsson et al., 1999).

Culturing Protocols. ZoBell and Morita in the year 1957 were among the first to attempt to isolate barophilic bacteria. However, the first pure culture isolates of barophilic bacteria were reported in 1979 (Yyanos, 1979). The overall methodology for isolation of deep-sea bacteria involves the collection of deep-sea samples and their transfer into pressure vessels with appropriate medium. The cells are then subcultured onto low-melting-point agar medium under high pressure. The isolation of cells that grow better under high pressure is a test of barophily. Barophiles can be isolated using a silica gel pour tube method (Deming, 1985) using agar plates pressurized with an oxygen–helium mixture. A dilution-to-extinction procedure (Deming et al., 1988) can also be employed for culturing barophilic organisms, but the technique is limited by being able to isolate only dominant bacteria (Nakayama et al., 1994). Table 2.8 highlights important media recipes utilized for culturing barophiles. Nakayama et al. (1994) isolated deep-sea microorganisms by the modified Dorayaki method. Two planoconvex-lens-shaped small marine agar plates are prepared and streaked. The streaked side is covered with another marine agar plate, which, in turn, is placed between sterile retortable pouch films that are heat-sealed and placed in a pressure vessel.

Limitations. Although exploration of the deep sea and its denizens is of great value to industrial biotechnology, attempts to characterize these organisms have been hampered by the lack of appropriate methodologies to grow them (Straube et al., 1990). The pressure response of these deep-sea dwellers are temperature dependent (Marteinsson et al., 1999). Sample collection using oceanographic vehicles, storage in anaerobic conditions in glass syringes, and enrichment culture under in situ pressure conditions are difficult and costly tasks.

2.3. EXTREMOLYTES

Extremophilic microbes harbor in their cells biological molecules to sustain the extremes of survival. These biological molecules, called *extremolytes*, can be accumulated by microorganisms either by de novo synthesis or by uptake from the environment (Lentzen

TABLE 2.8. Barophiles Isolated to Date with Their Habitat and Culture Media Composition

Organism	Habitat	Type	Media	References
Strains DB21MT-2, DB21MT-5	Unmanned deep-sea submersible Kaiko in the Mariana Trench, Challenger Deep, at a depth of 10,898 m	Obligate barophile: 70 MPa for strain DB21MT-2 and 80 MPa for strain DB21MT-5	^a Marine broth 2216 (Difco)	Kato et al., 1998
<i>Thermococcus barophilus</i> sp. nov.	Mid-Atlantic Ridge depth, 3550 m by American submersible <i>Alvin</i>	barophile	^b YPS-20 (Marteinsson et al., 1999) or modified thermotoga (Huber et al., 1986)	Marteinsson et al., 1999
<i>Moritella</i> sp. strain 2d2	Intestinal contents of deep-sea fishes	Barophilic bacteria	Marine broth (Difco)	Nakayama et al., 1994
Bacterial strains DB5501, DB6101, DB6705, and DB6906	Deep sea mud, Suruga Bay, 2485 m; Ryukyu Trench, 5110 m depth; Japan Trench, 6909 m depth	Barophilic bacteria	Marine broth 2216 (Difco)	Kato, 1995
<i>Desulfovibrio profundus</i> sp. nov.	Ocean Drilling Program Site 798B in the Japan Sea (water depth, 900 m)	Barophile	^c Postgate's sulfate-reducing bacterial medium	Bale et al., 1997
Bacterial strain DSS12	Ryukyu Trench, 5110 m depth	Moderately barophilic	Marine broth 2216 (Difco)	Kato, 1995
Bacterial strains DSK1 and DSK25	Japan Trench 6356 m; Japan Trench, 6500 m	Barotolerant	Marine broth 2216 (Difco)	Kato, 1995

^aFormula: peptone + yeast extract + ferric phosphate + artificial seawater (pH 7.5).

^bComposition (per litre of distilled water): 0.96 g PIPES buffer, 2.4 g NaCl, 0.16 g MgSO₄·7H₂O, 0.016 g NH₄Cl, 0.04 g CaCl₂·2H₂O, 0.16 g yeast extract (Difco), and 10 mM glucose.

^cComposition (per litre): 0.5 g of KH₂PO₄, 1.0 g of NH₄Cl, 1.0 g of CaSO₄, 5.0 g of MgSO₄·7H₂O, 3.5 g of sodium lactate (70% v/v solution) or 1.8 g of sodium acetate (anhydrous), 1.0 g of yeast extract, 0.1 g of ascorbic acid, 0.1 g of thioglycolic acid, 0.5 g of FeSO₄·7H₂O, 32.0 g of NaCl, 5.0 g MgCl₂·6H₂O, and 1.0 mL of resazurin (0.1%).

and Schwarz, 2006). Extremolytes stabilize biological macromolecular structures such as membranes, proteins, and nucleic acids under harsh environmental conditions such as salt concentrations above 1 M and temperatures above 80°C (Kumar et al., 2010). Most of the extremolytes or compatible solutes are restricted to thermophilic and halophilic prokaryotes. Some marine cyanobacteria are also a potent source of extremolytes such as dolstatins (Poncet, 1999).

Extremolytes are characterized by their low molecular mass and accumulation in response to stress, mainly salt and temperature stress, concentration rising to 1 to 2 M, depending on the extracellular osmolarity. Extremolytes can be grouped as amino acids, sugars, phosphorus-containing compounds, glyceric acid derivatives, and polyols. Table 2.9 shows the details of various extremolytes classified as pyrimidine derivatives, carbohydrate extremolytes, phosphodiester derivatives, bioactive compounds, and extremolytes from eukaryotes. The chemical structures of the various extremolytes are presented in Figure 2.5.

The application of extremolytes is vast and can be summarized for its use in the pharmaceutical and food industries. They also lead to stabilization of proteins and nucleic acids. Extremolytes such as Hydroxyectoines, which reduce the highly toxic side effects of immunotoxins when administered in combination with the immunotoxins (Barth, 2000), not only protect proteins from denaturation but DNA and cells as well. In contrast with mesophiles, which accumulate neutral or zwitterionic compounds, the solutes of hyperthermophiles are typically negatively charged. (2*R*)-2-(α -*D*-Mannopyranosyl)glycerate (abbreviated here as mannosylglycerate) is one of the most widespread solutes among thermophilic and hyperthermophilic prokaryotes (Faria et al., 2008).

2.3.1. Production and Purification of Extremolytes

Growth media composition, carbon source, agitation, temperature, and salinity are the potent selection factors for successful isolation of extremolytes. The growth phase at which the cells are harvested determines their final concentration. Extremolytes are secondary metabolites, hence need to be harvested between the middle and late exponential phase. They are highly soluble in water and accumulate in the cytoplasm (Santos et al., 2006). The extraction method of extremolytes should be effective within the concentration range in which they are present. Ethanol–chloroform extraction is more appropriate for low-molecular-mass extremolytes such as glycine betaine, trehalose, and taurine. HPLC (high-performance liquid chromatography), NMR (nuclear magnetic resonance), mass spectrometry, and TLC (thin-layer chromatography) can be directly conjugated with this extraction method for identification of the extremolytes.

An alternative approach can be recombinant production in host strains from the synthetic pathway genes by cloning and expression. This approach was chosen for mannosylglycerate, where the bifunctional mannosylglycerate synthase from *Dehalococcoides ethenogenes* was overproduced in *E. coli* and *Saccharomyces cerevisiae* (Empadinhas et al., 2004).

Halomonas elongate under high salt conditions (15 to 20% w/v NaCl) has been used in the manufacturing process for ectoines. Intracellular ectoines are obtained by osmotic down-shock treatment, and the product is purified further by electrodialysis, chromatography, filtration, evaporation, and crystallization (Lentzen and Schwarz, 2006), a process known as *bacterial milking*. Another process, called *permanent milking* (Lentzen and Schwarz, 2006) is now used by Bitop AG for the production of ectoines on a large scale

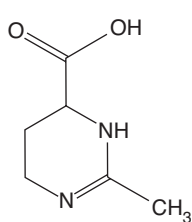
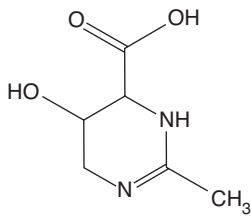
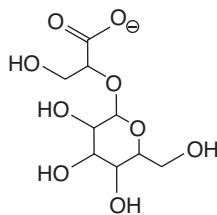
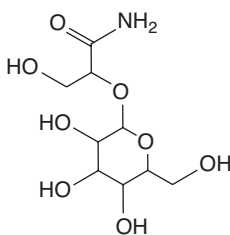
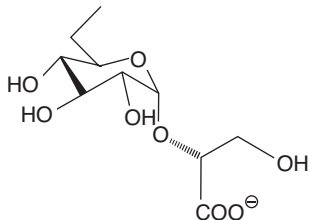
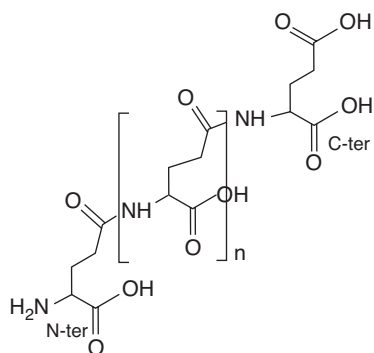
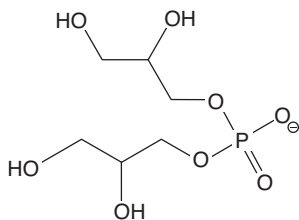
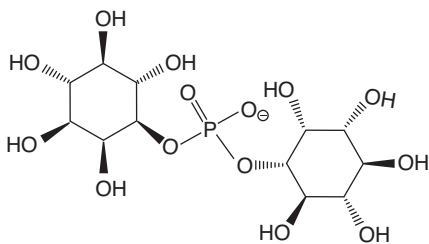
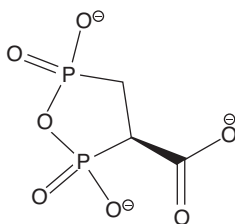
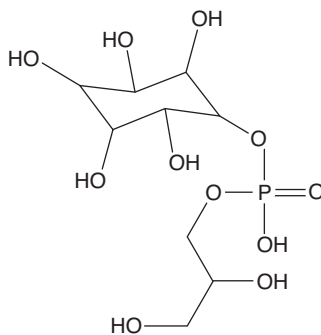
TABLE 2.9. Source and Properties of Extremolytes From Extremophiles

Extremolyte	Organism	Extremophilic Source	Roles	References
Hydroxyectoine	<i>Streptomyces</i> strain	<i>Pyrimidine Derivative</i> Thermophile	Protection of oxidative protein damage (LDH); reduction of VLS in immunotoxin therapy; stabilization of retroviral vaccines; induction of thermotolerance in <i>E. Coli</i> ; protection of <i>P. putida</i> against; anhydrobiotic stress	Kumar et al., 2010
Ectoine; (4S)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid	<i>Halorhodospira halochloris</i>	Halophile	Enzyme stabilization against heating, freezing, and drying; protection of LDH against heat and freeze-thawing; inhibition of insulin amyloid formation; stabilization of tobacco cells against hyperosmotic stress; block of UVA-induced ceramide release in human keratinocytes; protection of the skin barrier against water loss and drying out; protection of skin immune cells against UV radiation; reduction of UV-induced SBCs; prevention of UVA-induced photoaging; cytoprotection of keratinocytes	Galinski, 1985
Mannosylglycerate (firoin)	<i>Rhodothermus marinus</i>	<i>Carbohydrate Extremolytes</i> Thermophile	Stabilization of enzymes against thermal stress and freeze drying	Kumar et al., 2010
Mannosylglyceramide (firoin-A)	—	—	Stabilization of recombinant nuclease	

(continued)

TABLE 2.9. (Continued)

Extremolyte	Organism	Extremophilic source	Role	References
<i>Carbohydrate Extremolytes (continued)</i>				
Glucosyl glycerate	Halotolerant cyanobacteria	Halophile	Enzyme stabilizer under high temperature	Mackay et al., 1984
Poly- γ -glutamate	<i>Natrialba aegyptiaca</i>	Halophile	Nylon-like material as a versatile and chiral polymer	Yamasaki et al., 2010
<i>Phosphodiester Derivatives</i>				
α -Diglycerol phosphate	<i>Archaeoglobus fulgidus</i>	Halophile	Thermostabilization of proteins and rubredoxin protein	Kumar et al., 2010
Di-myo-inositol-1,1'-phosphate	<i>Pyrococcus furiosus</i> and <i>Thermotoga maritima</i>	Hyperthermophile	Thermostabilizers of enzymes	Scholz et al., 1992
Cyclic	<i>Methanothermobacter thermoautotrophicus</i>	Thermophile	Protection of plasmid DNA from hydroxyl radical	Kanodia and Roberts, 1983
2,3-diphosphoglycerate	<i>Aquifex pyrophilus</i>	Thermophile	Protein stabilization	Lamosa et al., 2006
Glyceryl-myo-inositol phosphate				
<i>Bioactive Compounds</i>				
Kahalalide F	<i>Elysia rufescens/Bryopsis</i> sp. (mollusk/green alga)	Halophile	Treatment of patients with severe psoriasis	Kumar et al., 2010
E7389	<i>Halichondria okadae</i> (sponge, synthetic)	Halophile	Treatment for breast cancer	Kumar et al., 2010
Curacin A	<i>Lyngbya majuscula</i> (cyanobacterium)	Halophile	Potent inhibitor of cell growth and mitosis	Kumar et al., 2010
<i>Extremolytes from Eukaryotes</i>				
Peloruside A	<i>Mycale</i> sp.	Marine sponges	Potent inhibitor of cell proliferation	Lentzen and Schwarz, 2006

**Ectoine****Hydroxyectoine****Firoin****Firoin A****Glucosyl glycerate****Poly-γ-glutamate****α-Diglycerol phosphate****Di-myo-inositol-1,1'-phosphate****Cyclic 2,3-diphosphoglycerate****Glyceryl-myo-inositol phosphate****Figure 2.5.** Two-dimensional chemical structures of extremolytes.

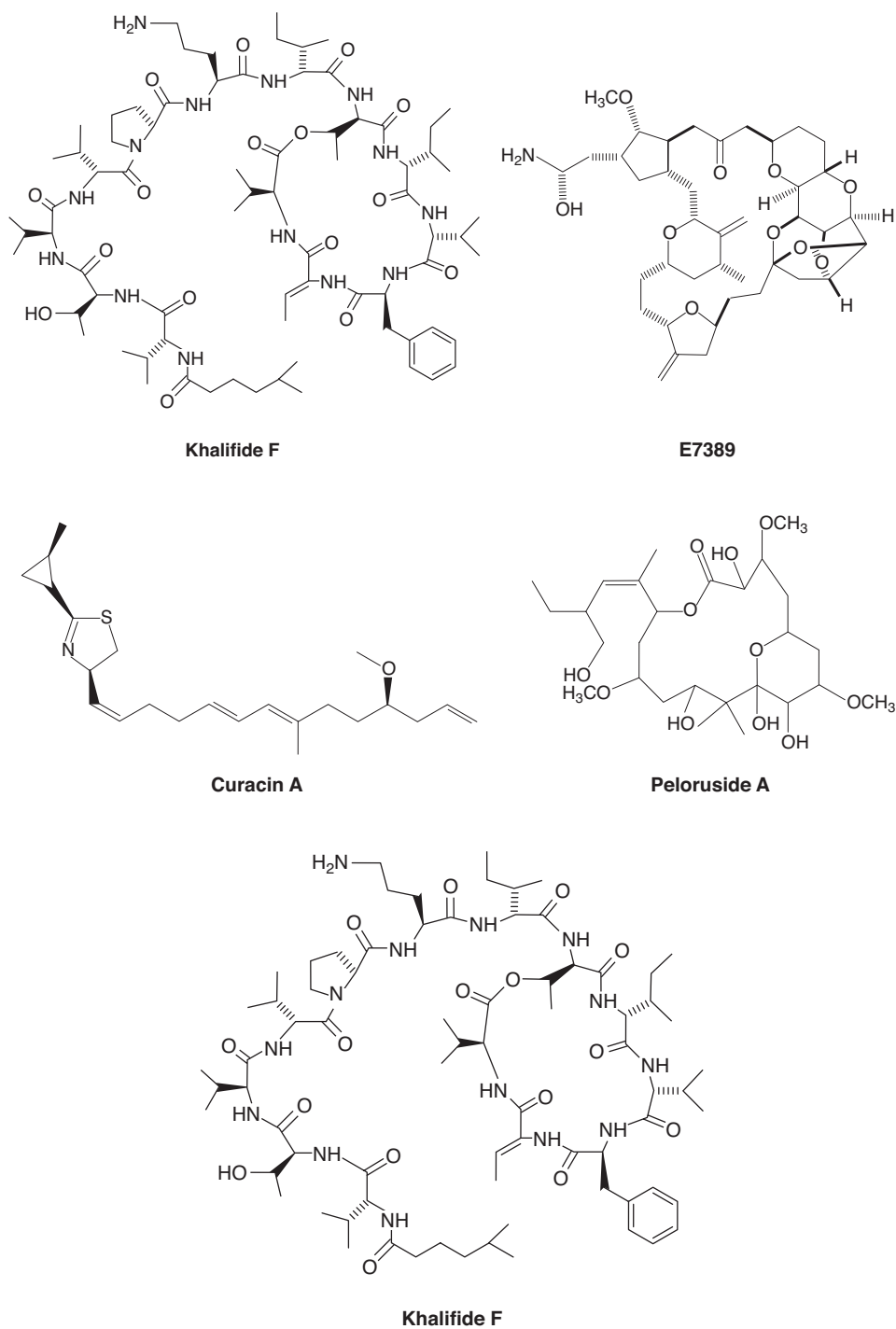
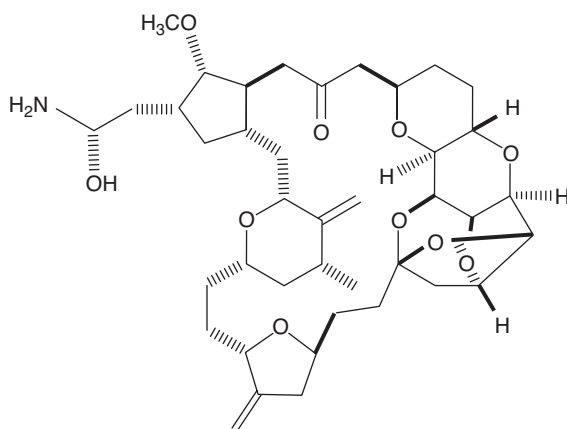
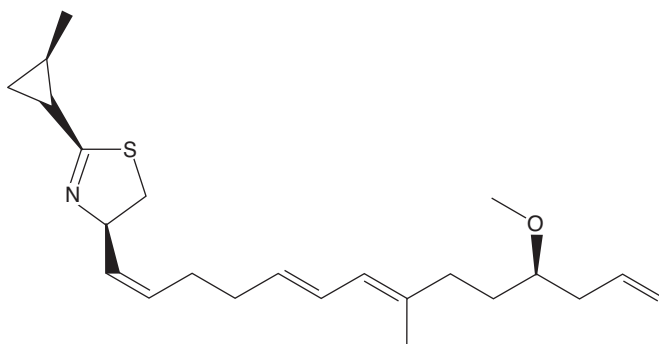
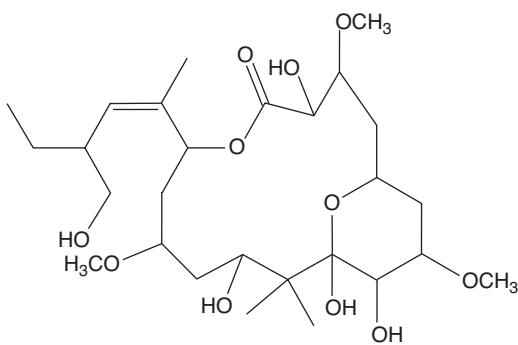


Figure 2.5. (Continued)

**E7389****Curacin A****Peloruside A****Figure 2.5.** (Continued)

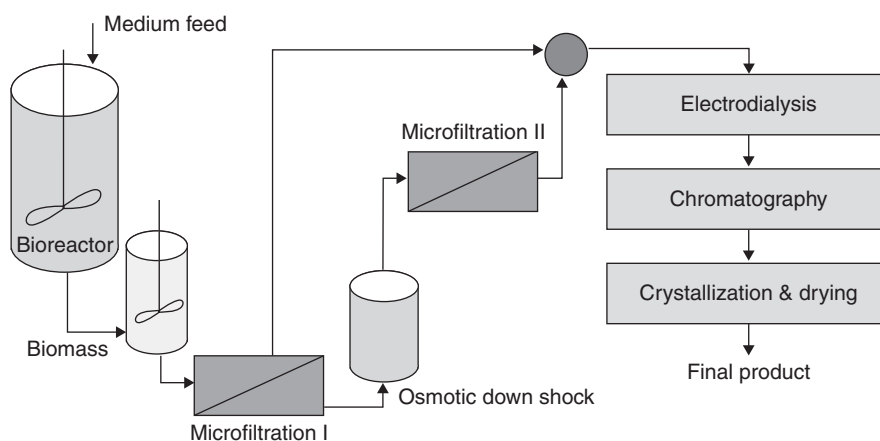


Figure 2.6. Ectoine productions by the bacterial milking process. [Adapted from Lentzen and Schwarz (2006), with permission from Springer Science+Media.]

(Fig. 2.6). Bitop AG also produces pure hydroxyectoines and glucosyl glycerol on a large scale. Extremolytes are accumulated at a high level, up to 25% of cell dry mass (Lentzen and Schwarz, 2006), making large-scale production feasible. The ectoines were the first extremolytes to be produced on a large scale. zDIP was produced by fermentation of *Pyrococcus furiosus* at 98°C under anaerobic conditions (Lentzen and Schwarz, 2006). Extremolyte production in recombinant hosts (e.g., *E. coli* or yeast) is more practical and realistic. The availability of the synthetic pathway genes for extremolyte production marks the success of this approach. Mannosylglycerate was overproduced in *E. coli* and *S. cerevisiae* by cloning the mannosylglycerate synthase gene from *Dehalococcoides ethenogenes* (Empadinhas et al., 2004). Cyclic 2,3-diphosphoglycerate was produced in a recombinant *E. coli* harboring the genes for 2-phosphoglycerate kinase and cyclic 2,3-diphosphoglycerate synthetase (Moritz, 1975).

Chemical synthesis is another approach for production of the osmolytes (Santos et al., 1998). α -Diglycerol phosphate was synthesized by this approach. This robustness of *Halomonas elongate*, together with the ability to achieve high cell densities, has led to its use in the manufacturing process for ectoines. The industrial bioprocess bacterial milking was designed for the large-scale production of ectoine by Sauer and Galinski (1998). In this process, *Halomonas elongate* is cultivated under high-salt conditions and ectoines are intracellularly accumulated. They are recovered by applying an osmotic shock which leads to the opening of mechanosensitive channels in the inner membrane of *H. elongate* (Lentzen and Schwarz, 2006). The product solution is further purified by electrodialysis, chromatography, filtration, evaporation, and crystallization. Ectoine had even higher productivity, on a metric ton scale, when subjected to the continuous fermentation process permanent milking developed recently (Lentzen and Schwarz, 2006). Hydroxyectoine was produced on an industrial scale (13.5% of the cellular dry weight) by mass cultivation of *Marinococcus* strain M52 by fed-batch strategy, with media exchange. Hydroxyectoine was extracted by Soxhlet extraction with methanol by osmotic down-shock by washing with demineralized water (Frings et al., 1995). Removal of salt by a simple washing procedure of whole cells (down-shock) is usually connected to a decrease in osmotic pressure inside the

cells (Frings et al., 1995). Alternatively, hydroxyectoine can be produced with *Halomonas elongate* by changing the fermentation conditions in the bacterial milking process (Lentzen and Schwarz, 2006).

2.3.2. Detection, Identification, and Quantification of Extremolytes

NMR is an ideal choice for the identification, characterization, and quantification of extremolytes, as the technique can be used for structural identification and solute quantification. To date, all novel solutes have been discovered in extremophiles using this technology (Santos et al., 2006). ¹H, ¹³C, and ³¹P NMR are carried out for cell extracts, and results are compared with those reported in the literature for identification of the compound. Two-dimensional NMR followed by mass spectrometry are used for determining the structure and molecular mass of unknown compatible solutes. The most commonly used spectra are correlation spectroscopy (COSY), nuclear Overhauser effect spectroscopy (NOESY), total correlation spectroscopy (TOCSY), heteronuclear single-quantum correlation spectroscopy (¹H/¹³C HMQC), heteronuclear multiple-bond correlation spectroscopy (¹H- and ¹³C-HMBC), and ³¹P spectrum. Galinski (1991) identified and characterized a novel osmolyte, *N*-α-carbamoyl-L-glutamine 1-amide from the halophilic phototrophic bacterium *Ectothiorhodospira marismortui* by NMR and mass spectrometry. Further purification of extremolytes are brought about by simple and well-known chromatographic techniques such as TLC, ion-exchange chromatography, and size-exclusion chromatography. The solvent systems used to separate extremolytes are given in Table 2.10.

2.3.3. Limitations

The limitations in attaining extremolytes are in culturing their extremophilic sources in fermentors with high cell densities, as described earlier. The accumulation of extremolytes is dependent on the type and growth condition of extremophiles. Often, a mixture of several osmolytes is produced and their intracellular concentration can range from millimolars to

TABLE 2.10. Solvent Systems Used to Separate Compatible Solute in Thin-Layer Chromatography

Solvent System	Type of Compounds Separated
<i>Silica Gel</i>	
Chloroform:methanol/acetic acid	Sugars
Glacial acetic acid/H ₂ O (30 : 50 : 8 : 4 v/v/v/v)	
Butanol/pyridinal water (7 : 3 : 1 v/v /v)	Sugar polymers
Butanol/ethanol/water (5 : 3 : 2 v/v/v)	Phosphosugars and sugars
<i>n</i> -Propanol/ammonium 25% (1 : 1.5 v/v)	Alcohol phosphodiesteres
Phenol/water (4 : 2 v/v)	Betaines
<i>Cellulose Paper</i>	
Acetonitrile/0.1 M ammonium acetate (6 : 4, v/v), pH 4.0	Amino acids and related compounds

Source: Adapted from H. Santos, P. Lamosa, and N. Borges, *Methods in Microbiology*, Vol. 35, with permission from Elsevier. Copyright © 2006.

1 to 2 M. Moreover, complex growth media contravene de novo synthesis of extremolytes (Santos et al., 2006). An associated problem involves the recovery of extremolytes from cultures of extremophiles due to the accumulation of glycine betaine, which reduces the intracellular concentration of de novo synthesized extremolytes (Frings et al., 1995). To overcome such a problem, a glycine betaine-free medium would be the best choice. To prevent glycine betaine accumulation, 1% of liquid fish peptone can be used instead of yeast extract in medium, as it contains only a small quantity of glycine betaine.

2.4. CONCLUSIONS

Recent research in extremophilic microorganisms highlight promising new and innovative technologies for the isolation and characterization of extremophiles. One of the most notable is a solid culture technique using a porous solid plate made of nanofibrous cellulose was developed which can be used for a solid culture of wide varieties of extremophiles because it retains its integrity even under extreme physicochemical conditions, such as high temperatures or low or high pH values. The same group also used a centrifuge successfully as an incubator for culturing microbes where the gravity is more than 400,000 times that of Earth. Extremophilic culture collections are developing such as the novel CAMR Culture Collection (Centre for Applied Microbiological Research, Porton Down) of thermophilic microorganisms. The collection comprises over 2000 thermophilic organisms that have been isolated from environmental samples obtained from around the world over the past 25 years. The Culture Collection of Cryophilic Algae (CCCRyo) in Potsdam-Golm near Berlin is focused on “snow algae” from polar and alpine environments. The CCCryo serves as a biore-source of extremophiles for public and industrial research. Strains can be ordered via these websites, which can be accessed through <http://cccryo.fraunhofer.de/web/infos/welcome>. One of the most interesting potential in developing extremolytes from extremophiles is cloning their genes in transgenic organisms and crop plants to increase their stress tolerance to salt. This approach has been carried out in model plants such as *Arabidopsis thaliana*, rice, and tobacco.

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STRATEGIES FOR THE ISOLATION AND CULTIVATION OF HALOPHILIC MICROORGANISMS

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3.1. INTRODUCTION

The world of halophilic microorganisms is highly diverse. Organisms adapted to life at salt concentrations up to saturation (>300 g/L total dissolved salts) can be found in each of the three domains of life: Archaea, Bacteria, and Eukarya. The crystallizer ponds of solar salterns found worldwide in coastal tropical and subtropical areas provide an excellent example: The main components of the brine ecosystem are typically *Haloquadratum walsbyi* and relatives (Archaea), *Salinibacter ruber* (Bacteria), and *Dunaliella salina* (Eukarya). A few phylogenetic lineages consist entirely of halophiles (operatively defined here as organisms that grow at optimal or nearly optimal rates above 100 g/L salt): the order Halobacteriales (Archaea, Euryarchaeota), the Halomonadaceae (Bacteria, Gammaproteobacteria), and the Halanaerobiales (Firmicutes). Many other highly salt-requiring and salt-tolerant microorganisms have close relatives adapted to life in freshwater or marine environments (Oren, 2002a,b, 2008).

Also, functionally, the halophiles are a highly diverse group. We know halophilic chemoheterotrophic aerobes and anaerobes living on a great variety of carbon and energy sources, oxygenic and anoxygenic photoautotrophs, photoheterotrophs, chemolithoautotrophs, organisms that live by anaerobic respiration using a variety of electron acceptors, fermentative organisms, and more. With a few exceptions (see also Section 3.4) most

metabolic types known from low-salt environments can also be encountered at salt concentrations approaching saturation. Table 3.1 lists representative examples of halophilic microorganisms, showing the diversity present in nature.

In many hypersaline environments the microorganisms are exposed to multiple forms of stress in addition to the high salt (NaCl) concentration present: We find halophiles in salty environments at high or low pH, high or low temperatures, and sometimes high concentrations of toxic divalent cations. Accordingly, the diverse world of the halophiles encompasses many *polyextremophiles* adapted to cope with more than one form of stress.

Compared to the alkaliphiles and the thermophiles, the halophiles are not exploited extensively in biotechnological applications. There are two notable exceptions: the production of β -carotene from *Dunaliella* and production of the compatible solute ectoine from *Halomonas*. Many more potential applications have been proposed, but these have yet to be materialized on a large scale (Oren, 2010). More applications may be discovered in the future; the world of halophiles is so diverse that many organisms with industrial potential are undoubtedly hiding in the brines and sediments of salt lakes and other hypersaline ecosystems.

In this chapter a number of case studies are presented that show how in recent years, novel and interesting halophilic microorganisms belonging to different physiological types and phylogenetic affiliations have been obtained from high-salt environments using a variety of isolation strategies. These examples not only illustrate the increase in the known diversity of halophiles in past decades, but also provide paradigms for the use of diverse approaches to the isolation of novel types.

3.2. THALASSOHALINE AND ATHALASSOHALINE HYPERHALINE ENVIRONMENTS

Brines formed by evaporation of seawater (*thalassohaline brines*) reflect the ionic composition of the marine environment, with Na^+ and Cl^- as the main ions and a near-neutral pH. A saturated solution of NaCl that forms a suitable medium for *Halobacterium* and many other extreme halophiles has a water activity (a_w) value of about 0.76. Many hypersaline environments are characterized by an ionic composition very different from that of seawater (*athalassohaline environments*) and sometimes with water activities lower than those of NaCl-based brines. Highly alkaline brines dominated by Na^+ , Cl^- , HCO_3^- and CO_3^{2-} and hardly any Mg^{2+} and Ca^{2+} , as found in soda lakes worldwide, are examples of such athalassohaline waters.

When evaporation of seawater leads to massive crystallization of NaCl as halite, such as occurs in saltern crystallizer ponds as well as in some natural environments, brines are formed that are rich in Mg^{2+} , a chaotropic ion that weakens electrostatic interactions and destabilizes biological macromolecules. The square halophilic archaeon *Haloquadratum walsbyi*, the main heterotrophic microorganism found in many crystallizer ponds (see also Section 3.3.1), is markedly magnesium tolerant (Burns et al., 2007) and has interesting adaptations to life at high magnesium concentrations and low water activities. These include the presence of energy-demanding cation efflux systems and also the relatively low A + T content in its DNA (47.9 mol%, far lower than that of all other members of the Halobacteriaceae), which may be an adaptation to life in high-magnesium environments, as in a highly stable high-GC genome, high magnesium might result in even greater DNA rigidity, which

TABLE 3.1. Examples of Halophilic Microorganisms of Different Phylogenetic Affiliation Displaying Different Types of Metabolism^a

Phylogenetic Affiliation	Name	Salt Tolerance and Optimum	Comments
<i>Archaea</i>			
Euryarchaeota, Halobacteriales	<i>Halobacterium salinarum</i>	Opt. 175–300 g/L	Aerobic heterotroph; can also obtain energy from light absorbed by bacteriorhodopsin
	<i>Haloquadratum walsbyi</i>	Opt. 180–300 g/L; requires at least 140 g/L salt	Aerobic heterotroph; unusually shaped flat, square cells (see Section 3.3.1)
	<i>Haladaptatus paucihalophilus</i>	Opt. 150–169 g/L; able to grow at 50 g/L NaCl and up to 300 g/L	Low-salt-requiring representative of the Halobacteriaceae (see Section 3.3.6)
	<i>Halorubrum sodomense</i>	Opt. 100–150 g/L NaCl, range 30–250 g/L	Highly magnesium-tolerant, magnesium-requiring organism from the Dead Sea (see Section 3.3.1)
	<i>Halorubrum lacusprofundi</i>	Opt. 150–200 g/L, range 90–300 g/L	Polyextremophile, can grow at 4°C (optimum 31–36°C)
	<i>Haloferax mediterranei</i>	Opt. 170 g/L, range 60–300 g/L	Aerobic heterotrophic with a large metabolic versatility; industrial potential for poly(β-hydroxyalkanoate) production
	<i>Halorhabdus tiamatea</i>	Opt. 270 g/L, range 100–300 g/L	Member of the Halobacteriaceae preferring an anaerobic lifestyle (see Section 3.3.4)
	<i>Natronomonas pharaonis</i>	Opt. 200 g/L, range 120–300 g/L	Polyextremophile, alkaliphilic member of the Halobacteriaceae growing optimally at pH 8.5–9.5 (see Section 3.3.2)
	<i>Halarchaeum acidiphilum</i>	Opt. 210–240 g/L, range 180–300 g/L	Polyextremophile, acidophilic member of the Halobacteriaceae, growing optimally at pH 4.4–4.5 (see Section 3.3.3)
	<i>Methanohalobium evestigatum</i>	Range 150–300 g/L	Anaerobic, methane producer
<i>Bacteria</i>			
Gammaproteobacteria Chromatiales	<i>Halorhodospira halochloris</i>	Opt. 140–270 g/L, range 100–350 g/L	Polyextremophilic anoxygenic phototroph, alkaliphilic, in which ectoine was first discovered

(continued)

TABLE 3.1. (Continued)

Phylogenetic Affiliation	Name	Salt Tolerance and Optimum	Comments
<i>Bacteria (continued)</i>			
Gamma-proteobacteria, Oceanospirillales	<i>Halomonas elongata</i>	Opt. 35–80 g/L, range 35–200 g/L	Aerobic heterotroph, used for the industrial production of ectoine
Gamma-proteobacteria	<i>Thiohalospira alkaliphila</i>	Opt. 120 g/L, range 30–230 g/L	Polyextremophile, obligately alkaliphilic, chemolithoautotrophic sulfur oxidizer
Gamma-proteobacteria, Desulfovibrionales	<i>Desulfohalobium retbaense</i>	Opt. 100 g/L, range 20–240 g/L	Obligately anaerobic sulfate reducer
Firmicutes, Clostridia, Natranaerobiales	<i>Natranaerobius thermophilus</i>	Opt. 3.3–3.9 M Na ⁺ , range 3.1–4.9 M Na ⁺	Polyextremophilic anaerobic haloalkalithermophile; opt. temp. 53°C, max. 56°C; pH 9.5 (see Section 3.3.5)
Firmicutes, Halanaerobiales	<i>Halanaerobacter salinaris</i>	Opt. 140–150 g/L, range 50–300 g/L	Fermentative obligate anaerobe, also capable of performing the Stickland reaction
	<i>Acetohalobium arabaticum</i>	Opt. 150–180 g/L, range 100–250 g/L	Homoacetogenic obligate anaerobe
	<i>Halothermothrix orenii</i>	Opt. 100 g/L, range 40–200 g/L	Polyextremophile, fermentative obligate anaerobe; opt. temp. 60°C, grows up to 68°C (see Section 3.3.5)
	<i>Selenihalanaerobacter shriftii</i>	Opt. 210 g/L, range 100–240 g/L	Obligate anaerobe; performs anaerobic respiration on selenate (see Section 3.5)
Haloplasmatales; remotely affiliated with the Firmicutes and the Mollicutes	<i>Haloplasma contractile</i>	Opt. 80 g/L, range 15–180 g/L	Obligate anaerobic; grows by fermentation or denitrification (see Section 3.3.4)
Gamma-proteobacteria, Methylococcales	<i>Methylohalobius crimeensis</i>	Opt. 58–87 g/L, range 12–150 g/L	The most halotolerant known among the aerobic methane-oxidizing bacteria (see Section 3.4)
Actinobacteria	<i>Actinopolyspora halophila</i>	Opt. 150–200 g/L, range >120–>300 g/L	The most halophilic known representative of the Actinobacteria
Bacteroidetes, Sphingobacteria	<i>Salinibacter ruber</i>	Opt. 150–300 g/L, range >150–>300 g/L	Red aerobic extremely halophilic nonarchaeal heterotroph (see Section 3.3.1)

TABLE 3.1. (Continued)

Phylogenetic Affiliation	Name	Salt Tolerance and Optimum	Comments
		<i>Eukarya</i>	
Algae, Chlorophyceae	<i>Dunaliella salina</i>	Up to 300 g/L	Oxygenic photoautotroph, used for β -carotene production
Fungi, Ascomycota, Dothideales	<i>Hortaea werneckii</i>	Opt. 60–180 g/L, grows slowly at 300 g/L	Aerobic heterotroph
Fungi, Basidiomycota, Wallemiales	<i>Wallemia ichthiophaga</i>	Needs 100 g/L, grows up to salt saturation	Aerobic heterotroph

^aEntries were selected as representative examples and selected on the basis of their special interest in the framework of this chapter. The table does not cover all groups in which halophiles are found.

may interfere with such essential processes as DNA replication and transcription (Bolhuis et al., 2006).

Also, some natural hypersaline lakes have elevated concentrations of magnesium. The Dead Sea, with 2 M Mg^{2+} and nearly 0.5 M Ca^{2+} , is an extreme case of a divalent cation–dominated athalassohaline brine (see also Section 3.3.2).

A water activity value of 0.60 to 0.61 is considered the lowest limit for life (Grant, 2004), but whether or not a hypersaline environment can support life also depends on the ionic composition, notably on the balance between stabilizing (*kosmotropic*) ions such as Na^+ and K^+ and destabilizing (*chaotropic*) ions such as Mg^{2+} and Ca^{2+} . The chaotropic action of magnesium and the presence or absence of sufficiently high concentrations of kosmotropic ions define the limit of life in high-magnesium brines. This was documented in a study of Discovery Basin, located at a depth of 3.58 km on the Mediterranean Sea floor off the western coast of Crete. The brines contain a gradient from 0.05 to 5.05 M MgCl_2 . The upper limit of MgCl_2 estimated to support life in the absence of compensating stabilizing ions is about 2.3 M. Some archaea can grow up to 2.5 M MgCl_2 , but only in the presence of significant concentrations of Na^+ (Hallsworth et al., 2007).

3.3. CASE STUDIES

3.3.1. Isolation of Aerobic Chemoheterotrophic Archaea from Solar Salterns

The crystallizer brines of solar salterns are generally colored reddish-pink as a result of the massive presence of pigmented members of the Halobacteriaceae. In addition, red-pigmented bacteria (*Salinibacter*) and unicellular eukaryotic algae (*Dunaliella salina*) may contribute to the color. Counts of prokaryotes are generally on the order of 10^7 to 10^8 mL^{-1} . Although solar salterns have already yielded many isolated halophilic microorganisms, archaea as well as bacteria, only a small fraction of the true diversity is known. Metagenomic 16S rRNA gene–based phylogenetic analyses of DNA extracted from such environments show an abundance of yet-uncultured phylotypes (see, e.g., Pašić et al., 2005; Park et al.,

2006; Ghai et al., 2011; Narasingarao et al., 2012). This does not mean that the organisms harboring those phylotypes cannot be cultured. The opposite is true. A study by Burns et al. (2004b) in an Australian saltern showed that most haloarchaeal groups detected are indeed cultivable. Using conventional plating techniques, more than 10^6 colony-forming units/mL were obtained. Such a result can only be achieved by using long incubation times (more than 8 weeks, which requires special provisions to prevent agar plates from drying out). The use of washed agar helped increase colony yield, but surprisingly, the nutrient composition of the medium had only a minor influence. The isolation of two elusive but particularly important and interesting prokaryotes from saltern crystallization ponds is discussed in further depth: *Haloquadratum walsbyi* and *Salinibacter ruber*.

***Haloquadratum walsbyi*.** To some extent it is surprising that the existence of the flat square archaeon now known as *Haloquadratum walsbyi* was not recognized until 1980. Walsby (1980) observed these unusually shaped organisms in a brine pool on the Sinai Peninsula (Egypt) and recognized their prokaryote nature because of the presence of gas vesicles. Subsequently, it was realized that these flat square cells are the most abundant type of prokaryotes in the brines of many saltern crystallizer ponds.

When culture-independent 16S rRNA sequence-based techniques of environmental genomics were first applied to saltern ponds, the most common phylotype found, originally designated the SPht phylotype, was only distantly related to those genera of Halobacteriaceae recognized at the time (Benlloch et al., 1995, 1996). Use of probes designed to react with that phylotype in fluorescence in situ hybridization (FISH) studies indicated that it belonged to Walsby's square prokaryote (Antón et al., 1999). Further culture-independent studies in which FISH was combined with autoradiography following incubation with radiolabeled substrates showed the square cells to use amino acids and acetate (Rosselló-Móra et al., 2003).

The elusive square archaeon was first isolated in 2004 (Bolhuis et al., 2004; Burns et al., 2004a). Growth media were based on low concentrations of organic nutrients (yeast extract, amino acids, acetate), addition of pyruvate as a preferred nutrient, and use of agarose rather than agar in solidified media. The fact that the square cells were present in higher numbers than other prokaryotes enabled the use of a "dilution to extinction" strategy to obtain the organism in pure culture (Burns et al., 2004a). The species was described as a new genus and species, *Haloquadratum walsbyi*, "the salt square of Walsby" jointly by the two groups (Burns et al., 2007). Analysis of its genome sequence identified some interesting features, such as a potential to take up dihydroxyacetone (a product of partial oxidation of glycerol excreted by *Salinibacter*; see below) and the possibility of using phosphonates as a phosphorus source. Theoretically, these properties could be used for the selective enrichment and isolation of *Haloquadratum* strains.

The commentary "Archaea with square cells" (Walsby, 2005) nicely summarizes the history of the discovery of *Haloquadratum* and all subsequent work done to elucidate the biology of one of the main players in the saltern ecosystem.

Salinibacter and Related Red Halophilic Members of the Bacteroidetes.

The first indications that a member of the bacteria, phylum Bacteroidetes, may occur in relatively large numbers (up to 15 to 20% of the total prokaryote numbers) in saltern crystallizer brines came from the application of FISH methodology: an abundantly present, slightly curved rod-shaped organism specifically stained with probes designed to detect

bacterial sequences (Antón et al., 1999). A survey of 16S rRNA gene sequences obtained from the microbial community led to the recognition that these “extremely halophilic bacteria” are phylogenetically affiliated with the Bacteroidetes. Growth experiments in which brine inocula were added to synthetic brine of 20, 25, and 30% salt supplemented with 0.1% yeast extract and with the community monitored with appropriate FISH probes showed that the bacterium also multiplies at the highest salinities previously thought to, almost exclusively, support growth of archaeal halophiles of the family Halobacteriaceae (Antón et al., 2000). FISH experiments combined with autoradiography showed that the organism takes up amino acids and acetate, but use of glycerol could not be confirmed (Rosselló-Móra et al., 2003). Later studies of the isolated organism proved that glycerol is used and is converted in part to dihydroxyacetone (Elevi Bardavid and Oren, 2008).

The organism was soon isolated, using two different methods. Both were based on growth of colonies on agar media with low concentrations of nutrients (yeast extract, amino acids). A few of the red–orange colonies that grew on the plates reacted with a 16S rRNA–directed FISH probe designed to detect the new phylotype. Thin-layer chromatography of the polar lipids extracted from randomly chosen colonies led to the identification of colonies of red prokaryotes that lack archaeal lipids and belong to the bacteria. The organism was described as *Salinibacter ruber* (Antón et al., 2002). It requires at least 15% salt, with optimal growth at 15 to 30%. Physiologically *Salinibacter* is surprisingly similar to *Halobacterium* and other haloarchaea, despite its disparate phylogeny.

A new genus, *Salisaeta*, related phylogenetically to *Salinibacter*, with the single species *Salisaeta longa*, was recently isolated from an experimental mesocosm filled with a mixture of water from the Dead Sea and the Red Sea. Like *Salinibacter*, its slender long rod-shaped cells are pigmented red, but with optimal growth at 10% NaCl + 5% MgCl₂ · 6H₂O, it is less salt requiring (Vaisman and Oren, 2009).

The differences in sensitivity toward antibiotics between *Salinibacter* and the halophilic Archaea were used to design selective enrichment and enumeration methods for *Salinibacter* and related halophilic bacteria. *Salinibacter* is not inhibited by anisomycin and by bacitracin, two antibiotics that inhibit growth of most or all members of the Halobacteriaceae (Elevi Bardavid et al., 2007). In field studies to assess the contribution of *Salinibacter* and halophilic archaea to the heterotrophic activities, taurocholate (causing lysis of most members of the Halobacteriaceae) and erythromycin (inhibiting protein synthesis by *Salinibacter*, and not affecting the archaea) are useful (Elevi Bardavid and Oren, 2008).

3.3.2. Magnesium-Requiring and Magnesium-Tolerant Archaea from the Dead Sea

The Dead Sea is an athalassohaline, slightly acidic (pH ~ 6) lake that becomes ever more stressful to life. The lake is drying out rapidly, and massive precipitation of NaCl as halite causes a relative increase in the concentrations of chaotropic divalent cations compared to kosmotropic monovalent cations. A comparison of the ionic concentrations in 1959–1960 (lower water layers) and 2007 shows an increase in Mg²⁺ and Ca²⁺ from 1.74 to 1.98 M, and from 0.43 to 0.47 M, respectively, an decrease in Na⁺ from 1.84 to 1.54 M, and a decrease in the molar ratio (Na⁺ + K⁺)/(Mg²⁺ + Ca²⁺) from 0.93 to 0.71. Most of the time the lake is virtually devoid of life, but dense microbial communities were observed in those rare cases when massive winter rain floods caused a significant dilution (10 to 15% at least) of the upper water layers, such as happened in 1980 and in 1992 (Oren, 1993).

Enrichment and isolation media for halophilic archaea from the Dead Sea often contained elevated concentrations of magnesium, but sodium concentrations much lower than generally used for the cultivation of Halobacteriaceae. Thus, *Haloferax volcanii*, isolated in the early 1970s, was described as a haloarchaeon with a moderate salt requirement and a high magnesium tolerance (Mullakhanbhai and Larsen, 1975). A thermodynamic analysis showed that the range of salt solutions supporting the growth of *Hfx. volcanii* differs greatly from that of a high-NaCl-adapted archaeon such as *Halobacterium salinarum*, both with respect to the water activity for optimal growth and the requirement for and tolerance toward divalent cations. *Hfx. volcanii* grows at water activities as high as 0.97, but never in the absence of magnesium, and does not grow below an a_w value of 0.77 (Edgerton and Brimblecombe, 1981). Use of high-magnesium growth media, combined with relatively low concentrations of organic nutrients (yeast extract, casamino acids, peptone) enabled the isolation of *Halorubrum sodomense* (Oren 1983) and *Halobaculum gomorrense* (Oren et al., 1995). *Hrr. sodomense* grows well in medium containing 0.8 M MgCl_2 + 2.1 M NaCl, and relatively good growth was even found in 1.5 or 2.0 M MgCl_2 in the presence of 0.5 M NaCl (Oren, 1983). *Hbc. gomorrense* grows optimally at 0.6 to 1 M Mg^{2+} in the presence of 2.1 M NaCl, and at least 1 M Na^+ is needed in the presence of 0.8 M MgCl_2 (Oren et al., 1995). The high-magnesium low-sodium environment of the Dead Sea also yielded isolates of a number of anaerobic fermentative bacteria, including *Halobacteroides halobius*, *Sporohalobacter lortetii*, and *Orenia marismortui* (Halanaerobiales).

Metagenomic studies were recently performed to learn more about the composition and functioning of the microbial community in the Dead Sea. The brine collected in 2007 contained a small but diverse community of archaea phylogenetically affiliated with the genera *Halorhabdus*, *Halosimplex*, *Halomicrobium*, *Halogeometricum*, *Haloplanus*, *Natronomonas*, and *Halalkalicoccus* (Bodaker et al., 2010). Most of these types still await isolation from the Dead Sea.

3.3.3. Isolation of Acidophilic Halophilic Archaea

Hypersaline acidic environments are rarely found. As stated above, the Dead Sea brine has a pH of around 6, but microorganisms isolated from the lake have their pH optimum in the neutral range. Only recently have truly acidophilic representatives of the Halobacteriaceae been isolated. Unexpectedly, the source material did not show an acidic reaction: It was a salt sample imported from Australia to Japan, to which powdered corals had been added (Minegishi et al., 2008). One isolate was recently described as a new genus and species: *Halarchaeum acidiphilum*. It grows in the pH range 4 to 6, with an optimum at pH 4.4 to 4.5 and 210 to 240 g/L NaCl (Minegishi et al., 2010).

3.3.4. Isolation of Unusual Anaerobic Halophiles from Deep-Sea Brines

The study of unusual, remote, and difficult-to-access environments can result in the isolation of unusual novel halophiles. Recent exploration of deep-sea brines on the bottom of the Red Sea and the Mediterranean Sea provides interesting examples.

Culture-independent studies of the microbial diversity at sites such as the Shaban deep and the Kebrit deep located at depths of 1.3 to 1.5 km deep on the bottom of the Red Sea and the Bannock basin, 3.3 km deep in the Mediterranean Sea, demonstrated the presence of a

wealth of novel lineages of bacteria and archaea (Eder et al., 1999, 2001, 2002; Daffonchio et al., 2006). These anoxic deep-sea brines have yielded cultures of two intriguing novel types of prokaryotes.

The first is the archaeon *Halorhabdus tiamatea*. It was recovered from the Shaban deep (salinity 24.4%). In contrast to nearly all other representatives of the Halobacteriaceae, it is unpigmented, which is not too surprising in an environment devoid of light. Whereas all other members of the family lead an aerobic lifestyle and have only limited abilities for anaerobic growth, *Hrd. tiamatea* preferentially lives anaerobically by fermentation. It only grows on complex substrates. Its mode of fermentation is yet unknown (Antunes et al., 2008a), but the analysis of its genome suggests that L-lactate may be one of the products (Antunes et al., 2011).

Even more unusual is *Haloplasma contractile*, a pleomorphic bacterium isolated from the Shaban deep that moves by contraction of tentacle-like protrusions. Phylogenetically, it belongs to a new lineage of the bacteria, between the Firmicutes and the Mollicutes, and it was classified in the new family Haloplasmataceae and the new order Haloplasmatales. It is an obligate anaerobe that grows at neutral pH between 15 and 180 g/L (optimum: 80 g/L, 30 to 37°C) and lives by lactate fermentation or by denitrification (Antunes et al., 2008b).

3.3.5. Isolation of Polyextremophilic Anaerobic Halophiles

Thermophilic Anaerobic Neutrophilic Halophiles. The most thermophilic among all known halophiles is an obligate anaerobe: *Halothermothrix orenii*, isolated from a salt lake in Tunisia. It grows optimally at 100 g/L salt and 60°C, tolerates up to 200 g/L salt, and can grow at temperatures up to 68°C (Cayol et al., 1994). Analysis of its genome (Mavromatis et al., 2009) did not show the abundance of highly acidic proteins characteristic of other members of the Halanaerobiales, to which it belongs phylogenetically. Its mode of osmotic adaptation remains to be elucidated.

Thermophilic Anaerobic Alkaliphilic Halophiles. Even more intriguing than the neutrophilic *Halothermothrix* are the haloalkalithermophilic anaerobes isolated from the Wadi Natrun, Egypt. The Wadi Natrun, located in the Sahara Desert 80 km northwest of Cairo, contains seven large shallow hypersaline and highly alkaline lakes and a number of ephemeral pools. In the past the Wadi Natrun lakes have yielded interesting microorganisms such as the aerobic alkaliphilic archaeon *Natronomonas pharaonis* and the haloalkaliphilic anoxygenic phototrophs *Halorhodospira abdelmalekii* and *Halorhodospira halochloris*. The recently discovered haloalkalithermophiles are phylogenetically affiliated with the class Clostridia, and were classified in a new order, the Natranaerobiales, and a new family, the Natranaerobiaceae. Three representatives of the group have been described. *Natronaerobius thermophilus* grows optimally at 53°C at pH 9.5 and at a total Na⁺ concentration of 3.3 to 3.9 M (range: 3.1 to 4.9 M). It ferments sucrose to acetate and formate. *Natronaerobius trueperi* produces lactate and acetate from pyruvate, requires 3.7 to 5.4 M Na⁺, and grows optimally at pH 9.5 and 52°C. *Natronovirga wadinatrunensis* grows optimally at pH 9.9 at 51°C in the presence of 3.7 to 3.9 M Na⁺ (Mesbah et al., 2007a,b; Mesbah and Wiegel, 2008; Bowers et al., 2009).

The combination of the extremes of high salt, alkaline pH, and elevated temperature may approach the physicochemical boundary for life. The existence of such haloalkalithermophiles raises intriguing questions about the bioenergetics of these organisms and the

ways they adapt to life at multiple forms of stress. An in-depth discussion is outside the scope of this chapter.

3.3.6. Isolation of Halophilic Microorganisms Associated with Plants and Animals

Halophilic microorganisms are sometimes found in unexpected habitats, and some of these are associated with plant and animal life. Some plants that grow on saline soils in arid areas excrete salts from glands on their leaves. A survey of the prokaryotes isolated from the phylloplane of the halophytic plant *Atriplex halimus* showed the presence of a diverse community of salt-tolerant bacteria, most of them pigmented (Simon et al., 1994). More in-depth studies, including culture-independent 16S rRNA gene-based work, were made on the microbial communities on the leaves of the tamarisk tree (*Tamarix* spp.). As a biotope for microorganisms, this environment is highly dynamic: The salinity increases as a result of evaporation during daytime, and the pH of the excreted brine can be high as well (Qvit-Raz et al., 2008).

The nasal cavities of a desert iguana contain salt glands excreting a concentrated solution of KCl. *Gracilibacillus dipsosauri* was isolated from this interesting environment. It can grow in 2.5 M KCl or 2 M NaCl. It is not a true halophile, as its optimal growth is between 0 and 1 M salt (Deutch, 1994). *Halococcus* spp. isolated related to *Hcc. dom-browskii* were found in the salt-excreting glands in the nostrils of the gull-like seabird *Calonectris diomedea* (Brito-Echeverría et al., 2009). Unlike most other members of the Halobacteriaceae, *Halococcus* cells do not lyse at low salt concentrations, and therefore the isolation of *Halococcus* spp. from Mediterranean seawater far from coastal areas is not too surprising (Rodríguez-Valera et al., 1979). Halophiles may well get dispersed to new habitats by such migrating birds.

3.3.7. Isolation of Halophilic Archaea from Low-Salt Environments

Typically, salt concentrations of 100 to 150 g/L are required for structural stability and viability of members of the Halobacteriaceae. Exposure to lower concentrations, even for short periods, leads to denaturation of the cells' proteins, including the glycoprotein cell wall present in most species, and the cells lyse.

Still, there are reports of the isolation of Halobacteriaceae from low-salt environments, and some members have a surprising ability to survive exposure to low salt for prolonged periods. Isolates affiliated with the genera *Halococcus*, *Haladaptatus*, and *Halogeometricum* obtained from a traditional Japanese salt field survived prolonged suspension at 50 g/L salt, and cells of an isolate related to *Haladaptatus paucihalophilus* (see below) even retained viability after 9 days at 30 g/L (Fukushima et al., 2007).

Sediments of the salt marshes and tidal creeks of the Colne Estuary (UK), with salinities around 35 g/L, yielded cultures belonging to three phylogenetic lineages of the Halobacteriaceae, and some even grew at 25 g/L NaCl (Purdy et al., 2004). Another marine and slightly hypersaline environment that yielded halophilic archaea (*Haloferax* and *Halococcus* spp.) is Hamelin pool, Western Australia, noted for its living stromatolites (Leuko et al., 2007).

Another low-salt habitat from which Halobacteriaceae were recovered is Zodletone spring, a sulfide-rich spring in Oklahoma. The spring waters have a low salinity (7 to

10 g/L), but far higher salt concentrations were measured in the adjacent soils. Following the recovery of 16S rRNA gene sequences attributed to halophilic archaea (Elshahed et al., 2004a), different isolates were obtained from the site: *Haladaptatus paucihalophilus*, which can grow at NaCl concentrations as low as 47 g/L (optimum: 150 to 180 g/L) (Savage et al., 2007); *Halosarcina pallida*, which grows at salt concentrations between 75 and 250 g/L with an optimum at 200 g/L, dies in 10 to 30 g/L salt but can be recovered after 24 h in 40 g/L salt (Savage et al., 2008); and several *Haloferax* strains, such as *Haloferax sulfurifontis*, which grows at NaCl above 60 g/L, lyses slowly at 30 g/L, and can survive for prolonged periods at 40 to 50 g/L (Elshahed et al., 2004b).

An even more unusual low-salt habitat in which Halobacteriaceae were detected is the geothermal vents of Kamchatka, Hawaii, New Mexico, California, and Wyoming. Strains related to *Haloarcula* were recovered from steam emerging from the fumaroles. Surprisingly, these isolates survived exposure to 75°C for 5 to 30 minutes (Ellis et al., 2008).

Finally, there are reports of the possible presence of archaea of the family Halobacteriaceae in the human gastrointestinal tract. 16S rRNA gene sequences related to *Halorubrum* and *Halobacterium* were recovered, albeit at a low abundance, from the intestinal mucosa of patients with inflammatory bowel disease. Successful enrichment of such archaea was reported in a medium with only 25 g/L NaCl, as shown by fluorescence in situ hybridization (Oxley et al., 2010). This shows that viable archaea may be recovered from mucosal tissue samples when enriched at relatively low salt concentrations, and that these organisms may be active in the human colon. Whether such organisms are transients in the intestine or regular members of the community is not yet clear. Culture-independent 16S rRNA-based studies of fecal samples from Korean people showed sequences related to *Halorubrum alimentarium*, *Halorubrum koreense*, *Halococcus morrhuae*, and *Halorubrum saccharovororum*. *Hrr. alimentarium* and *Hrr. koreense* were first isolated from salt-fermented seafood made from shrimps, a product also added to kimchi, a traditional Korean food. Halophilic archaeal species ingested with salt and fermented foods may thus survive in the digestive system (Nam et al., 2008).

3.4. THE UPPER SALINITY LIMITS OF DIFFERENT TYPES OF ENERGY GENERATION

Some microbial processes function well in hypersaline environments up to NaCl saturation, but certain types of energy metabolism were never shown to occur at elevated salinities. Examples of processes apparently absent at salt concentrations above 150 g/L are sulfate reduction with acetate as electron donor, methanogenesis by reduction of carbon dioxide with hydrogen or by splitting of acetate, and autotrophic oxidation of ammonia and nitrite (nitrification). In contrast, oxygenic photosynthesis, anoxygenic photosynthesis, aerobic respiration, and denitrification proceed up to the highest salt concentrations, whereas fermentation, sulfate reduction with lactate as electron donor, and chemoautotrophic sulfur oxidation apparently have somewhat lower maximum salinities (Table 3.2).

A theory was proposed to explain why certain types of metabolism can proceed at the highest salt concentration and others cannot. The model is based on our understanding of the energy yield of the various types of dissimilatory metabolism and the modes of

TABLE 3.2. Upper Salt Concentration for Growth of Cultured Microorganisms Displaying Different Modes of Energy Generation

Mode of Energy Generation	Approximate Upper Salt Limit (g/L)	Species	Phylogenetic Affiliation
Oxygenic photosynthesis	300	<i>Dunaliella salina</i>	Algae, Chlorophyceae
Anoxygenic photosynthesis	300	<i>Halorhodospira halophila</i>	Gammaproteobacteria, Chromatiales
Aerobic respiration	300	<i>Halobacterium salinarum</i>	Firmicutes, Halanaerobiales
Denitrification	300	<i>Haloarcula marismortui</i>	Euryarchaeota, Halobacteriales
Sugar fermentation	300	<i>Halanaerobacter salinarum</i>	Firmicutes, Halanaerobiales
Arginine fermentation	300	<i>Halobacterium salinarum</i>	Euryarchaeota, Halobacteriales
Fermentation—Stickland reaction	300	<i>Halanaerobacter salinarum</i>	Firmicutes, Halanaerobiales
Chemolithoautotrophic sulfur oxidation	290	<i>Thiohalospira halophila</i>	Gammaproteobacteria
Methanogenesis from methylated amines	250	<i>Methanohalobium evestigatum</i>	Euryarchaeota, Methanosarcinales
Homoacetogenic growth on $H_2 + CO_2$	250	<i>Acetohalobium arabaticum</i>	Firmicutes, Halanaerobiales
Sulfate reduction—incomplete oxidation	240	<i>Desulfohalobium retbaense</i>	Deltaproteobacteria, Desulfovibrionales
Sulfate reduction—complete oxidation of butyrate	230	<i>Desulfovermiculus halophilus</i>	Deltaproteobacteria, Desulfovibrionales
Aerobic methane oxidation	150	<i>Methylohalobius crimeensis</i>	Gammaproteobacteria, Methylococcales
Sulfate reduction—complete oxidation of acetate	130	<i>Desulfobacter halotolerans</i>	Deltaproteobacteria, Desulfobacterales
Methanogenesis from $H_2 + CO_2$	120	<i>Methanocalculus halotolerans</i>	Euryarchaeota, Methanomicrobiales
Chemolithoautotrophic ammonia oxidation	94	“Nitrosococcus halophilus”	Gammaproteobacteria, Chromatiales
Methanogenesis from acetate	~45	<i>Methanosarcina mazei</i>	Euryarchaeota, Methanosarcinales
Chemolithoautotrophic nitrite oxidation	<40	<i>Nitrococcus</i> sp. <i>Nitrospina</i> sp.	Gammaproteobacteria, Chromatiales; Deltaproteobacteria, Desulfobacterales

Source: Data from Oren (1999, 2011) and references therein.

osmotic adaptation used by different groups of microorganisms. It is based on the following assumptions (Oren, 1999, 2001, 2011):

- Life at high salt concentrations is energetically expensive.
- Bioenergetic constraints determine the upper salt concentration limit at which dissimilatory process can occur.
- The main factors that determine whether a certain type of microorganism can make a living at high salt concentrations are therefore:
 - The amount of energy generated during its dissimilatory metabolism.
 - The mode of osmotic adaptation used. Use of KCl as an intracellular solute is energetically more favorable than the biosynthesis of high concentrations of organic osmotic solutes.

This theory is there to be challenged, and therefore it is worthwhile to search for novel types of microorganisms that perform better at the highest salt concentrations than the most salt-tolerant microorganisms now known (Table 3.2). The finding of such organisms will not only increase our understanding of the functioning of hypersaline ecosystems, but also add interesting novel isolates for metabolic and bioenergetic studies. Notably, the following processes are of interest:

- Autotrophic nitrification. Oxidation of ammonia to nitrite was never shown to occur above 100 to 150 g/L salt, and the upper salinity limit for nitrite oxidation to nitrate may be even lower. This is understandable on the basis of the very low energy yield of the two reactions, as ammonia and nitrite are relatively oxidized substrates. In recent years little effort has been made to search for more halotolerant types of nitrifiers. The discovery of *Nitrosopumilus maritimus* and other archaeal autotrophic ammonia oxidizers may stimulate a renewed search for more halotolerant and/or halophilic types.
- Oxidation of sulfide and other reduced sulfur compounds is possible up to near-saturating salt concentrations, as could be expected on the basis of the relatively high energy yield of these processes. Thus, *Thiobacillus halophilus*, which grows optimally at 50 to 60 g/L salt, tolerates up to 240 g/L. In recent years, a number of haloalkaliphilic isolates were obtained from alkaline soils and soda lakes in Siberia, Egypt, the United States, and elsewhere: *Thioalkalibacter*, *Thioalkalivibrio*, *Thioalkalimicrobium*, and *Thioalkalispira* species (Sorokin and Kuenen, 2005a,b; Sorokin et al., 2006a,b; Banciu et al., 2008).
- The search for truly halophilic aerobic methane-oxidizing bacteria is still a major challenge. As aerobic methane oxidation is a highly exergonic process, there are no obvious thermodynamic reasons why methane cannot be oxidized at high salt concentrations (Oren, 1999). Moreover, methane is produced in many hypersaline anaerobic sediments from trimethylamine and other methylated amines, formed by, for example, the degradation of the osmotic solute glycine betaine. The most salt-tolerant methane oxidizer described is *Methylohalobius crimeensis*, isolated from hypersaline lakes in the Crimean Peninsula, Ukraine. Its optimum salt concentration for growth is 58 to 87 g/L NaCl, and only very slow growth was observed at 120 to 150 g/L (Heyer et al., 2005).

- Fermentation processes yield only small amounts of energy. However, the fermentative bacteria of the order Halanaerobiales employ the energetically favorable “salt-in” strategy by using KCl rather than organic solutes for osmotic balance. As a result, fermentation can function up to very high salt concentrations. For example, *Halanaerobacter salinarum* grows at 50 to 300 g/L NaCl with an optimum at 140 to 150 g/L. It gains energy by reduction of glycine betaine to trimethylamine with hydrogen or serine as electron donors or by the Stickland reaction (Mouné et al., 1999). As discussed in Section 3.3.5, the haloalkalithermophiles of the order Natranaerobiales need to cope with pH and temperature stress in addition to salt stress. Further studies of this intriguing group of bacteria are highly relevant to an understanding of the thermodynamics of life at the limits.
- Recent studies by Sorokin and co-workers (2008, 2010a,b, 2011) have greatly extended our understanding of the diversity of sulfate-reducing bacteria functioning at high salt concentrations, especially under alkaline conditions. Some novel isolates are surprisingly halotolerant in view of the low energy yield of the processes on which they make a living (Oren, 2011).
- Most biologically generated methane is formed by reduction of carbon dioxide with hydrogen or by the conversion of acetate to methane and carbon dioxide. These processes have never been shown to occur at elevated salt concentrations. The highest salt concentration at which methanogenesis from $H_2 + CO_2$ was documented is about 120 g/L, and the upper salinity for the acetoclastic split may be even lower.

3.5. FINAL COMMENTS

The world of halophilic microorganisms is extremely diverse, and novel types of halophiles are being described at an ever-increasing rate. A summary of a recently held international symposium on halophiles (Ma et al., 2010) shows clearly how much progress has been made in recent years. The discovery of *Salinibacter ruber* and the elucidation of its properties show that halophilic bacteria may play much larger roles at the highest salt concentrations than previously recognized. The fungi form another group whose importance in hypersaline environments has largely been ignored until recently. Now it has become clear that species of *Hortaea*, *Wallemia*, *Trimmatostroma*, *Debaryomyces*, and others can be found in hypersaline ecosystems worldwide, including salterns and salt lakes, thalassohaline as well as athalassohaline. Some can grow at salt concentrations of 250 g/L and higher (Butinar et al., 2005a,b; Zalar et al., 2005; Gunde-Cimerman et al., 2000, 2009). To what extent such fungi contribute to the heterotrophic activity in salterns and in other hypersaline ecosystems remains to be assessed.

When searching for novel types of halophiles that perform processes of interest, it of course makes sense to obtain samples from an environment where organisms with the desired properties are likely to be present. Thus, Mono Lake, California (ca. 90 g/L total salts; pH ~ 9.5), a hypersaline environment with a high content of arsenic compounds, was the place of choice to search for a haloalkaliphilic microorganism that not only tolerates high concentrations of arsenate, but was even claimed to use arsenic as a nutrient instead of phosphorus (Wolfe-Simon et al., 2011). Comparative genomics can also guide researchers in their search for suitable inocula to yield organisms with selected properties. Thus, a

comparison of the genomes of 10 Halobacteriaceae strains showed that glycosyl hydrolases, which may have a potential for use in biofuel production, are more likely to be found in halophiles isolated from soil or sediment (e.g., *Halorhabdus utahensis*, *Haloterrigena turkmenica*) than in those from brines (Anderson et al., 2011).

On the other hand, there was no a priori reason to assume that the sediments of the Dead Sea may harbor a selenate-reducing halophilic anaerobe such as *Selenihalanaerobacter shriftii* (Halanaerobiales) (Switzer Blum et al., 2001), as there are no records of high selenium concentrations there. Thus, interesting novel halophilic microorganisms may well be found in unexpected places!

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HALOPHILIC PROPERTIES AND THEIR MANIPULATION AND APPLICATION

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4.1. INTRODUCTION

Halophilic archaea and bacteria can be divided formally into two classes: extreme and moderate halophiles (Kushner, 1978, 1985; Larsen, 1986; Ventosa et al., 1998). Both can survive or even thrive in salty environments. These halophiles are thus used in certain commercial applications that require high salt concentrations, as summarized herein. They use different strategies to raise the internal osmotic pressure and cope with the external high salt concentrations. As the external salt concentrations are similarly high in the habitat of both halophiles, their extracellular proteins are intrinsically resistant to high salt concentrations. However, the cytoplasmic proteins are different between the two halophiles. The cytoplasmic proteins and enzymes of extreme halophiles, but not moderate halophiles, are also resistant to high salt concentrations. Thus, cytoplasmic proteins from extreme halophiles and extracellular proteins from both habitats have unique characteristics that make them salt-tolerant, i.e., a high acidic amino acid content (Lanyi, 1974; Oren et al., 2005). We review such unique characteristics of halophilic proteins based on our own studies using nucleoside diphosphate kinases (NDKs) from extreme and moderate halophiles.

Halophilic proteins have unique amino acid composition, as described above (Lanyi, 1974), which lead to novel properties of the proteins: slow refolding, resistance to organic solvents, and high aqueous solubility. Resistance to organic solvents finds some commercial applications of halophilic proteins. A slow, but reversible folding of halophilic proteins is reminiscent of natively unfolded proteins. The mechanism and utility of the organic-solvent resistance and slow refolding properties are discussed. High aqueous solubility found novel applications of halophilic proteins (i.e., a fusion partner for recombinant soluble expression). Attempts to develop such fusion vectors and use halophilic bacteria for soluble protein expression are also introduced in this review.

It would be of great commercial and academic interest if one could interconvert halophilic and nonhalophilic proteins or generate a hybrid with both halophilic and non-halophilic characters. We have observed that a halophilic *Halobacterium salinarum* nucleoside diphosphate kinase (HsNDK) is functional in low-salt solutions once folded at high salt concentrations (Ishibashi et al., 2001). Identification of a residue or residues that make this halophilic enzyme low-salt resistant may give us an idea of generating a hybrid enzyme having both characters. A second example involves interconversion of nonhalophilic and halophilic NDKs. Based on high sequence homology between halophilic *Halomonas* sp. 593NDK (HaNDK) and nonhalophilic *Pseudomonas aeruginosa* NDK (PaNDK) and the modeled atomic structure, we have mutated residues involved in oligomer assembly of the two NDKs above and observed that a single mutation can cause nonhalophilic tetrameric PaNDK to dissociate into the dimer and halophilic dimeric HaNDK to assemble into the tetramer (Tokunaga et al., 2008b) as reported in this review.

4.2. INDUSTRIAL APPLICATIONS OF HALOPHILIC ORGANISMS AND THEIR PROTEINS

Halophiles will have commercial applications in the processes where salts are required or naturally present. One obvious process might be production of salt. For many years, salt has been produced from seawater by evaporation (Baas-Becking, 1931). Halophiles that grow in this process have both positive and negative impacts on the quality of the salt produced (Jones et al., 1981; Javor, 2002). Halophiles play an essential role in various fermentation processes in salty environments (Margesin, 2001). These halophiles cause fermentation of ingredients, producing unique tastes, flavors, and aromas. A conventional pickle is produced by fermentation of plant products in the presence of salt at very high concentrations. Salty fermented products are popular in Asian countries (Roh et al., 2007). They are produced by fermentation of grains or meats in the presence of salt at high concentrations, hence utilizing halophiles. However, the type of bacteria and their role in these fermentation processes are little understood.

Wastewater clarification and decontamination are one of the lifelines in modern society. When wastewater contains high salt concentrations, halophiles may have potential for water treatment. There are a number of processes that produce salty wastewaters. The food-processing industries generate a large volume of saline wastewater. For example, a salty condition is a convenient way to maintain sterility useful for food conservation but generates contaminated water rich in salt (Antileo et al., 1997; Vitolo et al., 1999). Production of β -carotene using halotolerant *Dunaliella* generates hypersaline wastewater (Santos et al., 2001). Such industries as pickling plants and tanneries also generate hypersaline water. The hypersaline wastewater contains not only salt but also organic materials, often

including toxic compounds. For this, several halophilic bacteria have been proposed or tested: *Halobacterium salinarum*, *Halomonas* spp., *Bacillus* spp., *Haloferax denitrificans* and *H. mediterranei*, and *Halanaerobium lacusrosei* (Oren, 2010).

Halophiles are also used as a manufacturing factory of certain compounds. One such successful example is production of β -carotene (Ben-Amotz et al., 1991; Borowitzka, 1999; Raja et al., 2007). Green algae, *Dunaliella salina* and *D. bardawil*, accumulate large amounts of this pigment under high salt concentrations. β -Carotene has antioxidant properties. Another obvious example is the production of osmolytes. Moderate halophiles accumulate compatible solutes, or osmolytes, to raise the internal osmotic pressure and thus can be used as a cell factory to make osmolytes. Certain osmolytes find biotechnological applications as formulation excipients for long-term storage of pharmaceutical proteins (Lee et al., 1973, 1975; Frigon and Timasheff, 1975; Lee and Timasheff, 1975; Volkin et al., 1993; Chen et al., 1994a,b; Chen and Arakawa, 1996; Lentzen and Schwarz, 2006). One of the osmolytes, ectoine, has been used to protect proteins and nucleic acids (Lippert and Galinski, 1992; Kolp et al., 2006). Ectoine is a common compatible solute in eubacteria and is produced by *Halomonas elongata* (Canovas et al., 1997; Sauer and Galinski, 1998). It should be mentioned that not only ectoine but also many other osmolytes confer similar protective effects on proteins and other macromolecules (Yancey et al., 1982; Arakawa and Timasheff, 1983, 1984, 1985; Yancey, 2005). In other words, the effects are not specific to the chemical structure of ectoine, but rather are nonspecific. It has been demonstrated that the thermodynamic interaction of these stabilizing osmolytes with macromolecules are unfavorable, meaning that they do not bind to the macromolecules (Gekko and Morikawa, 1981; Gekko and Timasheff, 1981; Lee and Timasheff, 1981; Na and Timasheff, 1981; Arakawa and Timasheff, 1982a,b, 1987; Gekko and Koga, 1983). Thus, there are two reasons why moderate halophiles use osmolytes: their stabilizing effect and noninteracting nature. Both properties should not interfere with the protein function.

Ectoine may have certain specific effects. It appears to counteract skin aging induced by ultraviolet irradiation and hence may be used as a cosmetic skin care product (Motitschke et al., 2000; Buenger and Driller, 2004). This effect was postulated to be due to stimulation of the immune system, which reduces sunburn. It also inhibits aggregation of β -amyloid, a causative protein in Alzheimer's disease. However, this effect may not be specific to ectoine: trehalose, another osmolyte, also has been implicated as an aggregation-suppressing osmolyte (Arora et al., 2004; Tanaka et al., 2004). Glycerol is also an osmolyte and can be produced intracellularly by the alga *Dunaliella* (Chen and Jiang, 2009). However, more cost-effective glycerol production makes this halophilic process less practical.

The halophilic archaeon *Haloferax mediterranei* produces a polymer, poly(β -hydroxyalkanoate) (Don et al., 2006; Quillaguaman et al., 2010), which can be used as a material for biodegradable plastics. The same bacteria also excrete large amounts of anionic polysaccharides that can be used as gelling agents and emulsifiers, or in enhanced oil recovery (Anton et al., 1988). In microbial enhanced oil recovery (Banat, 1995; Banat et al., 2000), the harsh, salty conditions of many petroleum reservoirs will require halophilic bacteria that produce surfactants and help reduce interfacial tension at an oil-rock interface (Martínez-Checa et al., 2002, 2007).

Certain bacteria from moderate halophiles can also be used as a factory for recombinant protein production, based on its unique cytoplasmic environments containing compatible solutes. It is an established concept that compatible solutes enhance protein stability and folding (Chow et al., 2001; Ou et al., 2002; Pradeep and Udgaonkar, 2004). For example, proteins that have marginal folding capacity can be forced to collapse by one of the

osmolytes, trimethylamine *N*-oxide (TMAO) (Baskakov et al., 1999; Uversky et al., 2001; Akolkar et al., 2008; Street et al., 2010). Osmolytes also increase the stability of proteins against heat or denaturant treatments (Yancey et al., 1982; Arakawa and Timasheff, 1983, 1984, 1985; Yancey, 2005). Recombinant protein production using halophilic bacteria is described in depth in a later section.

Enzymes and proteins from extreme halophiles have halophilic characters, regardless of their locations (cytoplasmic or extracellular), as both locations have high salt concentrations. Those from moderate halophiles are halophilic when occurring in extracellular or periplasmic location; their cytoplasmic proteins have varying salt resistance, depending on the cytoplasmic salt concentrations. The halophilic enzymes and proteins are resistant to high salt concentrations and hence may find commercial applications in reactions that require high salt concentrations. However, their commercial applications are far less than those of other extremophiles, such as thermophilic and alkaliphilic bacteria.

Enzymes from thermophilic bacteria and archaea are extremely stable against high temperature and usually also resistant to chemical denaturants, organic solvents, and extreme pH values (Leuschner and Antranikian, 1995; Friedrich and Antranikian, 1996; Niehaus et al., 1999). Thermophilic bacteria produce thermostable enzymes such as amylases, pullulanases, cellulases, xylanases, chitinases, and proteinases (Cowan et al., 1987; Takayanagi et al., 1991; Bronnenmeier et al., 1995; Huber et al., 1995; Rudiger et al., 1995; Tachibana et al., 1996; Zverlov et al., 1996; Jorgensen et al., 1997). Among them, Taq polymerase brought the most critical advance in molecular biology (Chien et al., 1976). Halophilic enzymes may have been more or less disregarded in industrial applications compared with thermophiles. Nevertheless, halophilic enzymes have certain advantages (Oren, 2010; Litchfield, 2011) in industrial use as follows. (1) Their function is more efficient at increasing salt concentrations, under which nonhalophilic microorganisms are unable to grow and survive. (2) At high salt concentrations, the samples of halophilic enzymes may not have to be purified or sterilized, as contaminating proteins or other biological molecules are nonfunctional. (3) Many of them can work, as expected, under low water activity conditions but for an unexplained reason can also function in organic solvents. (4) Some of them also function at elevated temperatures. α -Amylase from *Haloarcula* sp. strain S-1, halophilic archaeon, exhibited maximal activity in 4.3 M NaCl at 50°C and was stable in various organic solvents (i.e., benzene, toluene, chloroform, etc.) (Fukushima et al., 2005). A lipase from a moderately halophilic bacterium, *Salinivibrio* sp. strain SA-2, showed maximum activity at 50°C in 0.01 M CaCl₂ and was tolerant to a wide range of salt concentrations (0 to 3 M NaCl). Furthermore, it retained 90% of its activity even at 80°C for 30 min of incubation (Amoozegar et al., 2008). A serine protease from *Natronococcus occultus* was stable and active in a broad pH range (5.5 to 12), with an optimal activity at 60°C in 1 to 2 M NaCl (Studdert et al., 2001). A nuclease H from *Micrococcus varians* subsp. *halophilus* is used in commercial production of flavoring agent, 5'-guanylic acid, by degrading RNA at 60°C and 12% salt, under which contaminating 5'-nucleotidase exhibits negligible activity (Kamekura et al., 1982).

4.3. EXTREME AND MODERATE HALOPHILES AND THEIR PROTEINS

Halophilic archaea and bacteria are mainly separated into two groups, extreme and moderate halophiles. Moderate halophiles are adapted to varying salt concentrations, whereas

extreme halophiles are adapted to extremely high salt concentrations, even to the saturated salt concentration. Thus, the strategy for survival is different for these two groups. Extreme halophiles take a “salt in” strategy and accumulate salts in cytoplasm at high concentrations, whereas moderate halophiles take a “salt out” strategy and accumulate osmolytes in cytoplasm. Both salts and osmolytes help raise the cytoplasmic osmotic pressure against external high salt concentrations. Although halophilic proteins may require unique properties to function under high osmolality, the different survival strategies between moderate and extreme halophiles may necessitate different properties of their proteins. It is also expected that cytoplasmic and secretory proteins may differ in each group, as the salt concentration can differ significantly in cytoplasmic and extracellular environments. We have been studying halophilic proteins from both moderate and extreme halophiles. One of the novel findings from this study is high solubility and reversible folding of halophilic proteins. We are developing a soluble expression tag based on halophilic proteins, as described later.

Moderately halophilic bacteria can grow over a wide range of salinities (0.2 M ~ saturated salts), optimally at 0.5 to 2.5 M salts, as they take a “salt out” strategy (no accumulation of high concentration of salts in the cells) with synthesis or incorporation of organic compounds, called compatible solutes, in the cells (Ventosa et al., 1998). Thus, cytoplasmic environments of halophilic bacteria are significantly different from those of *Escherichia coli* and hence may provide an opportunity for the soluble expression of recombinant proteins. Compatible solutes, also called *osmolytes*, enhance the stability of the proteins and hence may increase the folding efficiency and/or protect proteins from degradation (Galinski et al., 1995; da Costa et al., 1998). This expression system is also described later.

The amino acid composition of halophilic enzymes is characterized by an abundant content of acidic amino acids, which confers to the halophilic enzymes extensive negative charges at neutral pH and results in high aqueous solubility. This negative charge prevents protein aggregation when denatured and thereby leads to highly efficient protein refolding. While active under varying salt concentrations, their properties are modulated by salt concentration. β -Lactamase isolated from *Chromohalobacter* sp. strain 560 is active at both low and high salt concentrations, while higher salt (2 M) concentration makes this protein more stable to thermal denaturation, and lower salt (0.2 M) causes the increased refolding efficiency from thermal denaturation (Tokunaga et al., 2004, 2006a). The moderate halophile may generate multi-stress-adaptable enzymes to persist under the wide range of salt concentration. In the following two sections we describe the modulation of certain halophilic characters by mutations.

4.4. GENERATION OF LOW-SALT STABLE EXTREME-HALOPHILIC PROTEINS

Cytoplasmic proteins from extreme halophiles are extreme in halophilic properties. This means that understanding the factors that make them halophilic may shed light on their unique natures and in turn may lead to the way that the halophilic enzymes and proteins of commercial values can be modulated. Here we use nucleoside diphosphate kinase (NDK) from an extremely halophilic archaeon, *Halobacterium salinarum* (Hs) (i.e., HsNDK), of which we have extensive knowledge, as a model protein. The primary sequence shows high similarity with the NDKs from *Archaeoglobus fulgidus* (58.9% identity), *Bacillus*

subtilis (57.7%), *Synechocystis* sp. (56.7%), *Staphylococcus aureus* (53.3%), and *Drosophila melanogaster* (51.0%) (FASTA), yet is characterized as a halophilic protein. The HsNDK contains a large number of acidic amino acids (23.0 mol%) (Ishibashi et al., 2001). These signify the extreme end of halophilic properties. We and others have hypothesized that high salt concentration neutralizes strong charge repulsions and enhances hydrophobic interactions (Lanyi, 1974; Ishibashi et al., 2003), both of which can facilitate the proteins to fold. When expressed in nonhalophilic *E. coli*, HsNDK was inactive, but, as expected, was refolded and hence activated by high salt concentration. Nevertheless, it retained nearly full activity even at 30°C in the absence of salt, once folded as above by high salt concentration (Ishibashi et al., 2001). Thus, understanding the mechanism by which salt activates the enzyme and the enzyme maintains the folded structure without salt may give us a clue as to the salt dependence of structure formation and stability of halophilic proteins, in particular for HsNDK.

Folding of HsNDK does occur salt-concentration dependently. Folding was extremely slow in low salt conditions and accelerated by high salt concentration (Ishibashi et al., 2003). Figure 4.1 shows salt-dependent activation of HsNDK. There appears to be a low level of activation at day 8 even with 1 M salt, below which essentially no activation was observed within the time frame of experiments (over 8 days). Although activation was significantly enhanced at 2 M, it did not reach the maximum level even at day 8. The effects of salt appear to level off around 3 M; at 3.8 M, salt appears to suppress activation, although only slightly. These results indicate that the activation energy is large in the absence of salt and is reduced greatly by increasing salt concentration. Such salt dependence can be explained readily from high net negative charges of the protein that must be largely screened. Insufficient hydrophobic amino acids may also have some contributions to the slow folding rate. Figure 4.2 shows the effect of TMAO at 4 M on refolding of the HsNDK. TMAO showed

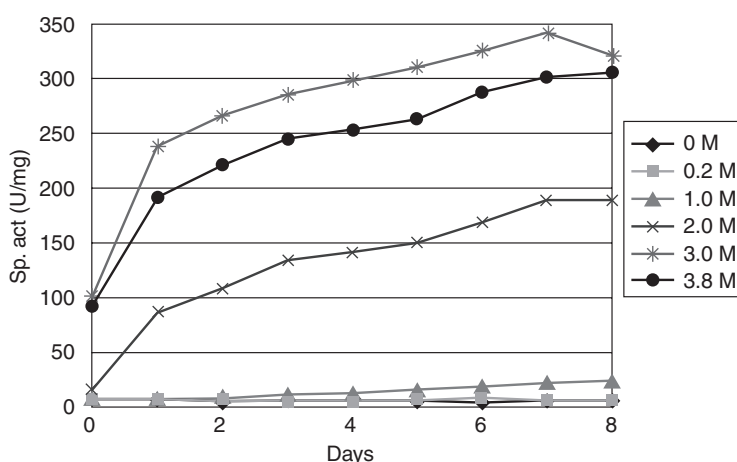


Figure 4.1. Time course of activation of HsNDK at various salt concentrations. HsNDK samples containing 6 M urea were dialyzed at 4°C against 50 mM Tris-HCl buffer (pH 7.5) containing respective concentrations of NaCl for 6 h. Immediately after the dialysis, HsNDK activity was assayed (shown at 0 day). Samples were incubated at 4°C, and HsNDK activity was assayed daily. The protein concentration in the refolding buffer was 74 µg/mL.

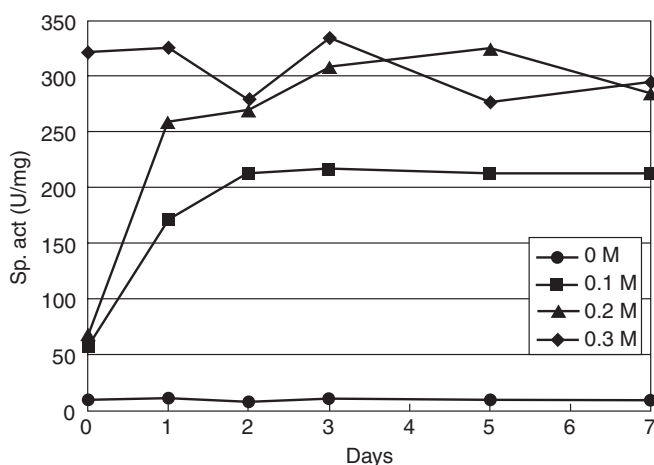


Figure 4.2. Time course of activation of HsNDK in the presence of 4 M TMAO at various salt concentrations. Urea-denatured HsNDK was dialyzed at 4°C for 6 h against 4 M TMAO/50 mM Tris-HCl buffer (pH 8.0) containing 0, 0.1, 0.2, and 0.5 M NaCl. Immediately after a 6-h dialysis, HsNDK activity was assayed (shown at 0 day). Samples were incubated at 4°C, and HsNDK activity was assayed daily.

no effect on folding without salt. However, 0.1 M salt, which was ineffective by itself (see Fig. 4.1), resulted in rapid activation when 4 M TMAO was present; there was immediate activation at day 0 and over 50% activation at day 1. The activation rate was enhanced further with increasing salt concentration to 0.2 and 0.3 M, far below the concentration required for refolding by salt itself; at 0.3 M, a maximum activation was already at day 0. The effect of TMAO is probably due to the enhancement of hydrophobic packing of the protein. Thus, it compensates for the insufficient hydrophobic amino acids in HsNDK and can refold the protein in the presence of charge shielding far less than required when TMAO was not present. The effect of salt in the absence of TMAO may then be conferred due to both charge shielding and enhancement of hydrophobic packing.

The results above clearly demonstrate that the activation barrier is too large for spontaneous folding in the absence of salt. The reverse may also be true; that is, unfolding reaction may also have a high energy barrier. Thus, although the native state is thermodynamically less stable in the absence of salt, the native structure and hence the activity of HsNDK may be protected by the high energy barrier once activated by high salt concentration, even after removal of salt. It should also be pointed out that folding and unfolding of HsNDK are fully reversible. There appears to be no off-pathway products during unfolding and refolding processes.

Even more surprising is the fact that HsNDK is a hexamer; thus, fully reversible refolding means that the subunit protein is soluble upon unfolding and forms native hexamer upon refolding. As shown in Figure 4.3A, the structure of each subunit is made of four antiparallel β -sheets ($\beta 1$, $\beta 2$, $\beta 3$, and $\beta 4$) flanked by interspersed α -helices ($\alpha 2$, $\alpha 6$, and $\alpha 8$) that form the core structure (Besir et al., 2005). This structure framework is very similar to the previously determined NDK structures for a hexameric human NDK-B (Morera et al., 1995). Thus, despite large differences in acidic amino acid content and hence net negative

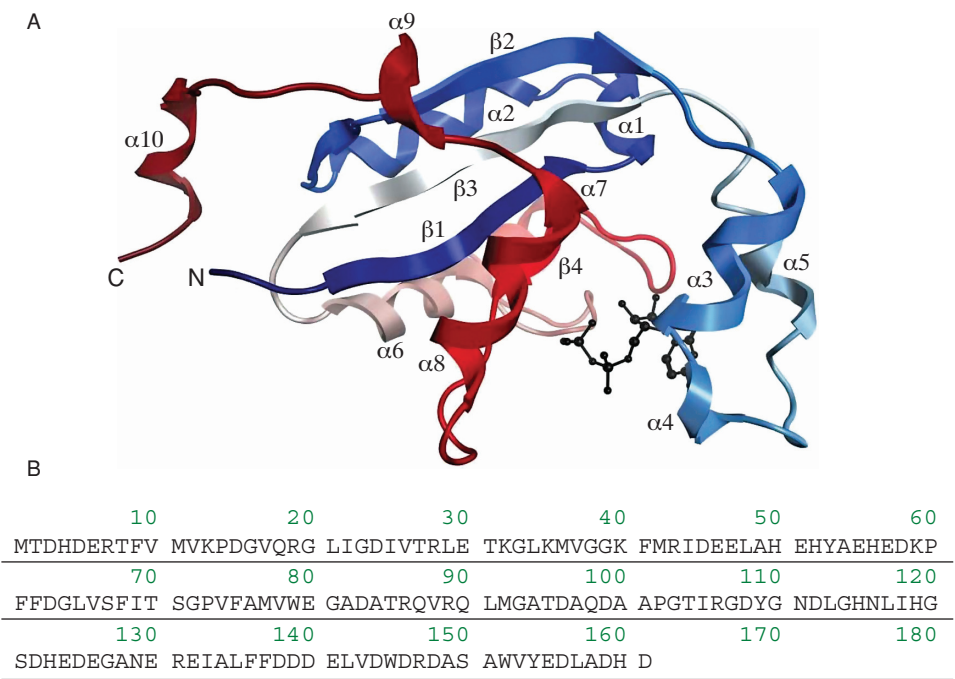


Figure 4.3. Structure (A) and sequence of HsNDK (B). The protein backbones of the monomer subunit are shown as blue to red from the N-terminus to the C-terminus (A). Chain termini and secondary structure elements were indicated (B). CDP was shown in a ball-and-stick representation. (See insert for color representation of the figure.)

charge, the structure of HsNDK is nearly identical to that of the human counterpart. This implies that the folding and stability of the HsNDK must overcome large electrostatic repulsions at the interface of the subunits, as described above.

HsNDK hexamer is made of the basic dimeric unit. Figure 4.4A and B show how the three dimeric units (green–yellow, red–brown, and blue–purple) assemble into the hexamer. To clearly see the interface between the monomers in the basic dimeric unit, the structure inside the box of Figure 4.4A is expanded in Figure 4.5A, in which $\alpha 2$ (in blue) and $\beta 2$ (in red) in yellow monomer generate a large contact surface area with $\beta 2$ (in blue) and $\alpha 2$ (in red) of green monomer. The $\alpha 2$ encompasses sequence from Gly21 to Glu30 and the $\beta 2$ from Met36 to Phe41 (see Fig. 4.3B for amino acid sequence). Such a large contact surface area is responsible for the stable dimeric structure, which may be maintained even when subjected to the thermal denaturation. These dimeric units assemble into the hexamer through various contacts. The structure inside the Figure 4.4B box illustrates the dimer–dimer interface, which is expanded in Figure 4.5B for the interface between the blue–purple dimer (right side) and the red/brown dimer (left side). Atomic packing is less dense than at the monomer–monomer interface (compare with Fig. 4.5A), suggesting weaker interactions of the dimer–dimer interface. Five amino acids constituting $\alpha 1$ (Asp15–Arg19, in green) of the red–brown dimer contact with Trp152 (in green) of the blue–purple dimer. Two Gln residues [i.e., Gln98 (in blue) from the red–brown dimer and Gln87 (in blue) from the

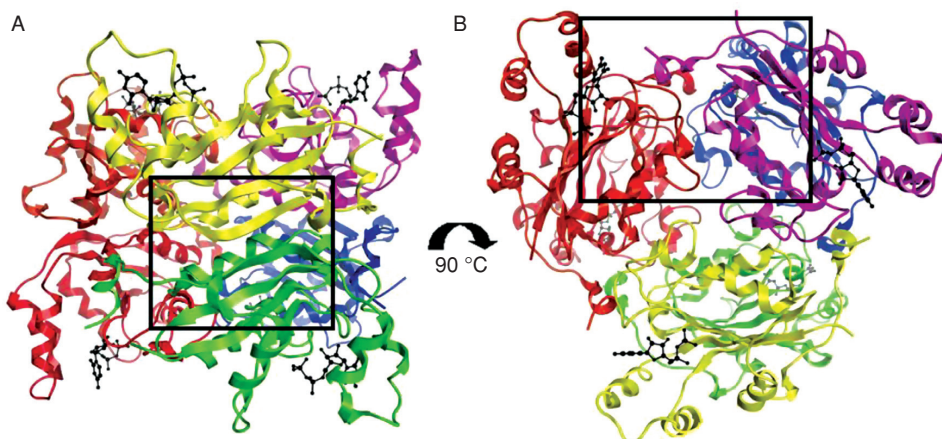


Figure 4.4. Hexameric structure of HsNDK; each monomer is depicted in different colors. Dual colors (e.g., red–brown, blue–purple) show basic dimeric units forming a hexamer. (A) Monomer–monomer interface (see the box); (B) dimer–dimer interface (see the box). (See *insert for color representation of the figure.*)

blue–purple dimer] contact each other. A loop (Gly107–His115) (in yellow) connecting $\alpha 7$ and $\beta 4$ of the red–brown dimer contacts with both the link (Thr31–Leu34) (in yellow) between $\alpha 2$ and $\beta 2$ and the C terminus (Trp152–Glu155, in yellow) of the blue–purple dimer. Trp152 (in green) of the blue–purple dimer is also involved in contact with the loop (Besir et al., 2005).

Can we generate a mutant of HsNDK: that is, a hybrid of HsNDK with both halophilic and nonhalophilic characters that refolds in low salt solution and hence has a lower activation energy than that of the parent HsNDK, based on the structure information? One residue that stands out in the structure is Gly 114, located in the dimer–dimer contact interface and interacting with Glu155 of the neighboring subunit in the hexameric structure of HsNDK. In Figure 4.5B, Gly114 is in $\beta 4$ of the red–brown dimer and Glu155 is at the C-terminus of the blue–purple dimer. This residue was mutated to Arg, which resulted in a spontaneous

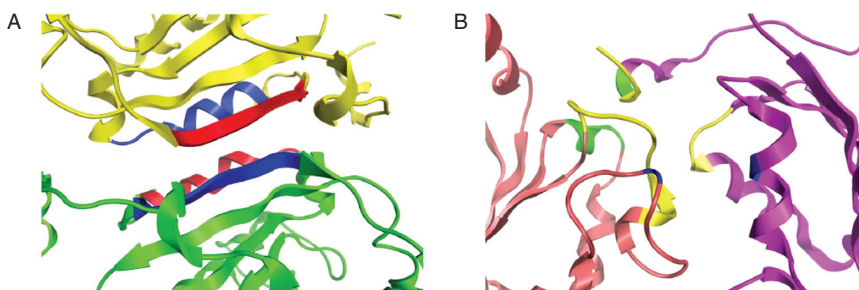


Figure 4.5. Detailed structure of monomer–monomer interface (A) and dimer–dimer interface (B). Contacts between two monomers (A) and between two dimers (B) are shown in color as described in the text. (A) Box in Figure 4.4A is expanded; (B) box in Figure 4.4B is expanded. (See *insert for color representation of the figure.*)

refolding at 1 M salt that was ineffective for the parent HsNDK (see Fig. 4.1). Furthermore, it resulted in about 10°C higher thermal stability in a low-salt solution than that of the parent molecule (Ishibashi et al., 2007). We thus speculated that the attractive electrostatic interactions occurred between Arg114 and Glu155 in G114R mutant and were reduced to the extent that the subunit assembly of this mutant protein can occur in low salt. To confirm this speculation, Gly114 was mutated to basic Lys (G114K), neutral Ser (G114S), or acidic Asp (G114D). The stability and refolding properties of G114S were similar to those of the parent HsNDK, as expected from a neutral-to-neutral substitution. G114K was similar to G114R, consistent with the speculation of the electrostatic stabilization between K(R)114 and E155. G114D lost activity, as this mutation caused dissociation into the dimers through enhanced charge repulsions (Ishibashi et al., 2009). These results suggest one factor that causes inefficient refolding in a low-salt solution: weak association between dimeric units to form a stable hexamer, as discussed above.

Halophilic malate dehydrogenase, a homotetrameric enzyme from *Haloarcula marismortui*, also unfolds at NaCl or KCl concentrations below 2 M, accompanied by dissociation into the dimer. This tetrameric enzyme is also made of two dimeric units with networks of complex interactions. The network is primarily through salt bridges associated with chloride binding sites (Madern and Ebel, 2007). Thus, it may be possible to produce a low-salt-resistant mutant by enhancing salt bridges without altered chloride bindings, which would enhance the dimer–dimer interactions, as in HsNDK.

Alkaline phosphatase (HaALP) derived from *Halomonas* sp. 593 is an important tool as a biological marker and for gene manipulation. This enzyme was inactive when expressed in *E. coli* but was converted to an active dimer when dialyzed against high NaCl concentration. It is interesting to note that other salts, such as KCl, were ineffective (Ishibashi et al., 2011b), suggesting that Na ion may be directly involved in monomer–monomer contact. In this case as well, mutations that can replace Na-mediated subunit interactions may confer HaALP low-salt resistance. Thus, one may be able to use this approach (i.e., enhancement of subunit–subunit interactions) to generate low-salt-resistant halophilic enzymes and proteins. Such enzyme and proteins should function under both low- and high-salt conditions. Even when the stabilization by mutation against low salt is insufficient, the enzymes may resist denaturation and inactivation against transient exposure to low-salt conditions.

It appears that N-terminal hexa-His-tag confers low-salt resistance to HsNDK without compromising its halophilic nature. As described above, recombinant HsNDK was expressed in *E. coli* as an inactive form. However, when expressed with an N-terminal hexa-His-tag form (HisNDK), HisNDK was active. In addition, refolding of heat-denatured HisNDK was much faster than HsNDK (i.e., no His-tag) (Ishibashi et al., 2004). As HisNDK was active and soluble at high salt concentrations, its halophilic nature has not been altered by the addition of His-tag. It has been suggested that several basic amino acids in the His-tag sequence interact with the negative charges on the native structure of HsNDK, stabilizing the native state. It appears that it stabilizes the hexameric structure of HsNDK by enhancing dimer–dimer interactions (Ishibashi et al., 2011a). If it is true, His-tag may restore the activity of G114D mutant, which lost the ability to assemble into the hexamer due to charge repulsion at dimer–dimer contact, as described earlier. Figure 4.6 shows the native-PAGE of HsNDK with His-tag (lane 1) and without His-tag (lane 2). As expected, unstable HsNDK dissociated into the dimer under the native-PAGE conditions (lane 2).

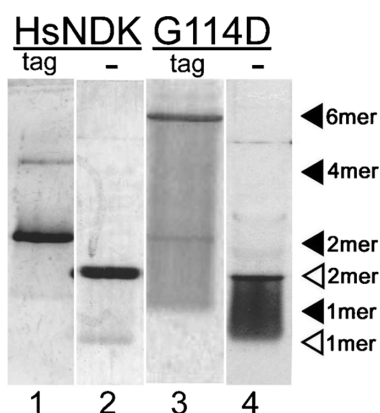


Figure 4.6. Native gel electrophoresis of HsNDK and G114D mutant with and without His-tag. NDK and G114D, with His-tag (tag) and without His-tag (–), were analyzed by native-PAGE. The assembly state of each band is shown next to the arrows, with His-tag (filled arrows) and without His-tag (open arrows), estimated by the relative mobility on native-PAGE.

Unexpectedly, more stable HisNDK also dissociated, although a faint tetramer band can be seen (lane 1); there was no detectable amount of hexamers. Thus, the condition for native-gel analysis appears to be strong enough to cause this stable HisNDK to dissociate. It is thus evident that His-tag has marginal stabilizing effects on HsNDK, although it can enhance folding under physiological conditions. The effect of His-tag was stronger on G114D than on the parent HsNDK, as there was no detectable dimer band with His-tag G114D (lane 3) compared with the major dimeric band for G114D without His-tag (lane 4): HisG114D was, instead, primarily the hexamers (lane 3). The observed strong effect of His-tag on G114D mutant indicates that the favorable electrostatic interactions between His-tag and D114 overwhelm the unfavorable charge repulsion between two dimers.

We conclude this section by stating that the low-salt resistance of halophilic enzymes and proteins may be modulated by altering subunit–subunit interactions. Mutations of charged amino acids or their additions may be one approach that can be taken to alter electrostatic interactions between the subunits.

4.5. INTERCONVERSION OF HALOPHILIC AND NONHALOPHILIC PROTEINS

A number of enzymes from halophiles have been purified and characterized. These enzymes are generally inactivated and denatured in the absence of salt (Eisenberg et al., 1992; Madern et al., 2000). That is, halophilic enzymes normally function under salty conditions. In this section we focus on the conversion of nonhalophilic enzyme into the new protein carrying halophilic properties through a protein engineering approach. Here we use NDK from a moderate halophile, *Halomonas* sp.593, as a model for interconversion between nonhalophilic enzyme and halophilic counterpart. NDKs from eukaryotes, archaea, and bacteria have been investigated extensively (Lascu, 2000; Lascu and Gonin, 2000). All NDKs have a common basic unit, dimer, which assembles into different oligomeric structures: for example, a hexamer of eukaryotes, archaea, and gram-positive bacteria, or a

tetramer of gram-negative bacteria (Janin et al., 2000; Lascu et al., 2000). To our surprise, the NDK from *Halomonas* sp. 593 (HaNDK) was a dimer (Yonezawa et al., 2007). It was the first report that a dimeric NDK is the native state. An active dimer structure of *Chromohalobacter salilexigens* DSM3043 NDK has been observed as a second example (Tokunaga et al., 2010a).

4.5.1. Dimer–Tetramer Conversion of HaNDK and PaNDK

The halophilic HaNDK has a high sequence homology with NDK from nonhalophilic *Pseudomonas aeruginosa* (PaNDK). Despite their high sequence homology, they show completely different subunit structure. HaNDK forms a homodimer in contrast to tetrameric structure of PaNDK. An intriguing question is whether there is a particular amino acid that determines their oligomeric structures. The first approach was to generate chimeric molecules of HaNDK and PaNDK: that is, Ha/Pa-chimeric NDK composed of the N-terminal half portion of HaNDK (N-terminal 70 residues) followed by the C-terminal half portion of PaNDK (71th to 143th residues) and Pa/Ha-chimeric NDK composed of the N-terminal half portion of PaNDK (N-terminal 70 residues) followed by the C-terminal half portion of HaNDK (71th to 141th residues). Fortunately, both genes to be combined had only one *CpoI* site at the middle of each gene (Tokunaga et al., 2006b). Figure 4.7A shows SDS-PAGE analysis of cross-linked chimeric NDKs (lanes 3, 5, 7, and 9). Upon cross-linking (marked +), PaNDK showed a ladder up to tetramer size (lane 3), an indication of its tetrameric size. The formation of a ladder corresponding to monomer, dimer, and trimer is due to inefficient cross-linking reactions. Conversely, HaNDK showed a ladder up to dimer (lane 5), consistent with its molecular weight. Pa/Ha chimeric NDK showed a characteristic dimeric band (lane 9), as in HaNDK (lane 5), suggesting that the C-terminal half of HaNDK determines its dimeric structure. Conversely, Ha/PaNDK (lane 7) and PaNDK (lane 3) showed a ladder of multiple bands up to the size of tetrameric molecules, indicating that Ha/PaNDK formed a tetramer, as in PaNDK. This implies that the C-terminal half of PaNDK determines its tetrameric structure. Thus, in both HaNDK and PaNDK, their C-terminal region contains critical residues that determine the oligomeric assembly. In addition, Figure 4.7A shows different migrations of these NDKs on SDS-polyacrylamide gel

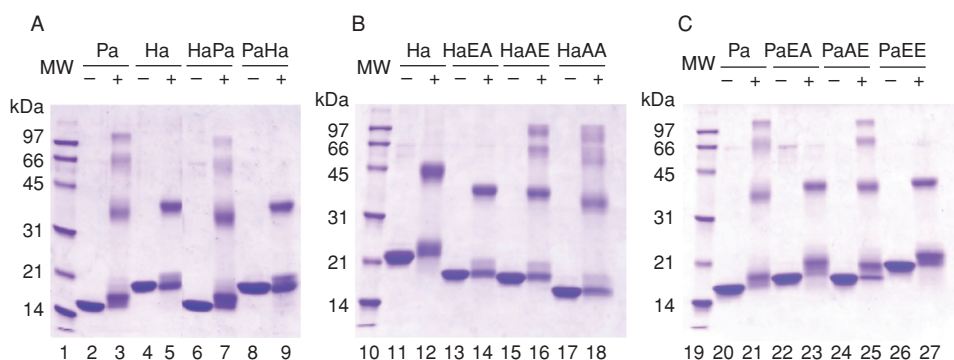


Figure 4.7. SDS-PAGE of PaNDK and HaNDK and their mutants with and without cross-linking. All samples were cross-linked as described by Tokunaga et al. (2008) and analyzed by SDS-PAGE. Samples with cross-linking (+) and without cross-linking (–) were shown.

(marked — for no cross-linking treatment, lanes 2, 4, 6, and 8) despite their similar molecular masses. HaNDK (lane 4) and Pa/Ha-chimeric (lane 8) NDK show a slower migration than PaNDK (lane 2) and Ha/Pa-chimeric NDK (lane 6); abnormal slow mobility is one of the hallmarks of halophilic proteins. These results suggest that the chimeric Pa/HaNDK is halophilic and the chimeric Ha/PaNDK is not, at least based on certain criteria, SDS-PAGE mobility and oligomeric structure. According to these criteria, having nonhalophilic PaNDK sequence at the C-terminal region makes the NDK nonhalophilic, and having a halophilic HaNDK sequence at the C-terminal region makes the NDK halophilic.

The results above suggest that dimer–dimer contact lies in the C-terminal region of the two NDKs. The crystal structure of tetrameric *Myxococcus xanthus* NDK (MxNDK) shows the location of the dimer–dimer interface. There are two contact regions in tetrameric MxNDK. The first region lies in Arg38 to Ser43 around the $\beta 2$ structure (i.e., the N-terminal region) and the second region lies in Ala130 to Glu137 at the C-terminal part (Williams et al., 1993). As described above, chimeric analysis suggested the importance of the C-terminal region in determining the oligomeric structure. The sequence of Ala130 to Glu137 of MxNDK is AYFFRETE and the corresponding sequence is AYFEESE in HaNDK and AYFFAATE in PaNDK. Comparison of these sequences shows striking differences at the 134th and 135th residues, as indicated above. Do these two amino acids determine the assembly of NDK protein? Thus, several proteins with mutations at these two residues were constructed. Namely, wild-type HaNDK/EE was mutated to three mutants, HaNDK/EA, HaNDK/AE, and HaNDK/AA, and wild-type PaNDK/AA was mutated to PaNDK/AE, PaNDK/EA, and PaNDK/EE. In this expression of NDK/XX, the first X after the slash represents the 134th residue and the second X represents the 135th residue, with the mutated residue underlined. Figure 4.7B (HaNDK mutants) and Figure 4.7C (PaNDK mutants) show the mobility of the wild-type and mutant proteins on SDS-PAGE. In the absence of cross-linking (–lanes), the double mutant, HaNDK/AA (lane 17), showed a faster mobility than the wild-type HaNDK/EE (lane 11), indicating loss of halophilic character. Similarly, the PaNDK/EE double mutant showed slower mobility (lane 26) than the wild-type PaNDK/AA (lane 20), indicating acquisition of halophilicity. The single mutants with AE or EA mutation (lane 15, 24 or lane 13, 22) showed intermediate mobilities and hence intermediate halophilic characters, all migrating between halophilic HaNDK and non-halophilic PaNDK. Cross-linking results are also shown in Figure 4.7B (HaNDK mutants) and Figure 4.7C (PaNDK mutants). HaNDK/EE (lane 12) and HaNDK/EA (lane 14) showed dimeric mobility, while HaNDK/AE (lane 16) and HaNDK/AA (lane 18) showed a ladder, an indication of a tetramer. Both PaNDK/AA (lane 21) and PaNDK/AE (lane 25) were also tetramers, indicating that the 134th Ala plays a major role in the tetramer formation. Two mutants having the 134th Glu, HaNDK/EA (lane 14) and PaNDK/EA (lane 23), showed the dimeric form.

The cross-linking results were confirmed by sedimentation velocity analysis (Tokunaga et al., 2008b). The size distribution analysis showed a dimeric molecular mass of HaNDK and a tetrameric molecular mass of PaNDK. Sedimentation velocity analysis also demonstrated that both HaNDK/AE and HaNDK/AA mutants were tetramers, consistent with the cross-linking results. Conversely, both PaNDK/EA and PaNDK/EE were converted to a dimer, also confirming the cross-linking results. It is evident from these data that the 134th residue makes a critical contribution to the subunit assembly as well as SDS-PAGE mobility. The Glu residue at this position directs dimer formation and the Ala residue leads to the formation of a tetramer, both in HaNDK and PaNDK. It appears that the 135th residue is less important.

A tertiary structure of wild-type HaNDK/EE was modeled based on the tetramer structure of MxNDK. The crystallographic structure of MxNDK revealed that the dimer–dimer contact surface area (estimated to be 473 Å² per monomer surface) is much smaller than the monomer–monomer contact surface area in the basic dimer unit (estimated to be 1092 Å²), similar to the HsNDK subunit–subunit interactions. This is probably the major reason for weaker association between two dimeric units to form a tetramer. If HaNDK/EE were to assemble into a tetramer as in MxNDK, the predicted dimer–dimer interface would have four negative charges, including Glu134. These negative charges perhaps prevented HaNDK from forming a tetramer. The importance of this region was also demonstrated from the structure analysis of *Chromohalobacter salexigens* NDK (CsNDK), which existed as an active dimer. Site-directed mutational analysis of CsNDK demonstrated that the two amino acids located at 134 and 136, not 134 and 135 in HaNDK, in the C-terminal region play a critical role in determining the tetramer assembly (Tokunaga et al., 2010a).

4.5.2. Generation of Halophilic PaNDK

As described above, halophilic HaNDK forms a dimer and mutation of Glu134 and 135 to Ala in HaNDK leads to a tetramer. Conversely, the nonhalophilic tetrameric PaNDK is converted to a dimer with both A134E and A135E mutations. Does this mean that the mutant tetrameric HaNDK/AA is nonhalophilic and the mutant dimeric PaNDK/EE is halophilic in every aspect? Typical properties of halophilic enzymes are summarized as follows: (1) higher optimal salt concentrations for enzyme activity, (2) higher reversibility to denaturation stress, (3) higher stability in the presence of salt, and (4) slower mobility on SDS-PAGE due to lower SDS binding (Tokunaga et al., 2006b). Wild-type HaNDK exhibits these characteristics.

The HaNDK double mutant, HaNDK/AA, migrates faster than the wild-type HaNDK on SDS-PAGE, as described above. Conversely, the PaNDK double mutant, PaNDK/EE, migrates slowly, just as does the wild-type HaNDK. In this case there is a correlation between an oligomeric structure (and hence mutation at these positions) and halophilicity: that is, the tetrameric HaNDK mutant is nonhalophilic and the dimeric PaNDK mutant is halophilic with respect to SDS-PAGE mobility. With regard to salt tolerance, the dimeric PaNDK/EE mutant showed salt-tolerant enzyme activity, with an optimal salt concentration at 50 mM, similar to the wild-type halophilic HaNDK. Conversely, the wild-type nonhalophilic PaNDK/AA and the tetrameric HaNDK/AA mutant were not salt tolerant, as they lost activity with increasing salt concentration. With regard to reversibility against heating, the wild-type PaNDK/AA formed aggregates and was hence irreversibly denatured, more so at higher protein concentration, by the heat treatment at 85°C for 5 min, as plotted in Figure 4.8A (right column, shaded bars). Conversely, the double mutant PaNDK/EE showed reduced activity loss upon heating under the same condition, regardless of protein concentration (right column, black bars) and behaved similarly to the wild-type HaNDK (left column, shaded bars). Thus, the substitutions of two Glu residues at residues 134 and 135 for two Ala convert nonhalophilic PaNDK/AA to a halophilic protein. However, the tetrameric HaNDK/AA still showed high reversibility regardless of the protein concentration (left column, black bars), inconsistent with its oligomeric structure and some nonhalophilic characters described above. The reversibility of HaNDK/AA was similar to that of the wild-type HaNDK/EE, suggesting that reversibility of heat denaturation depends on other factors. Thus, changes in oligomeric structure by mutation do not show an absolute correlation with one halophilic property: reversibility (Tokunaga et al., 2008a).

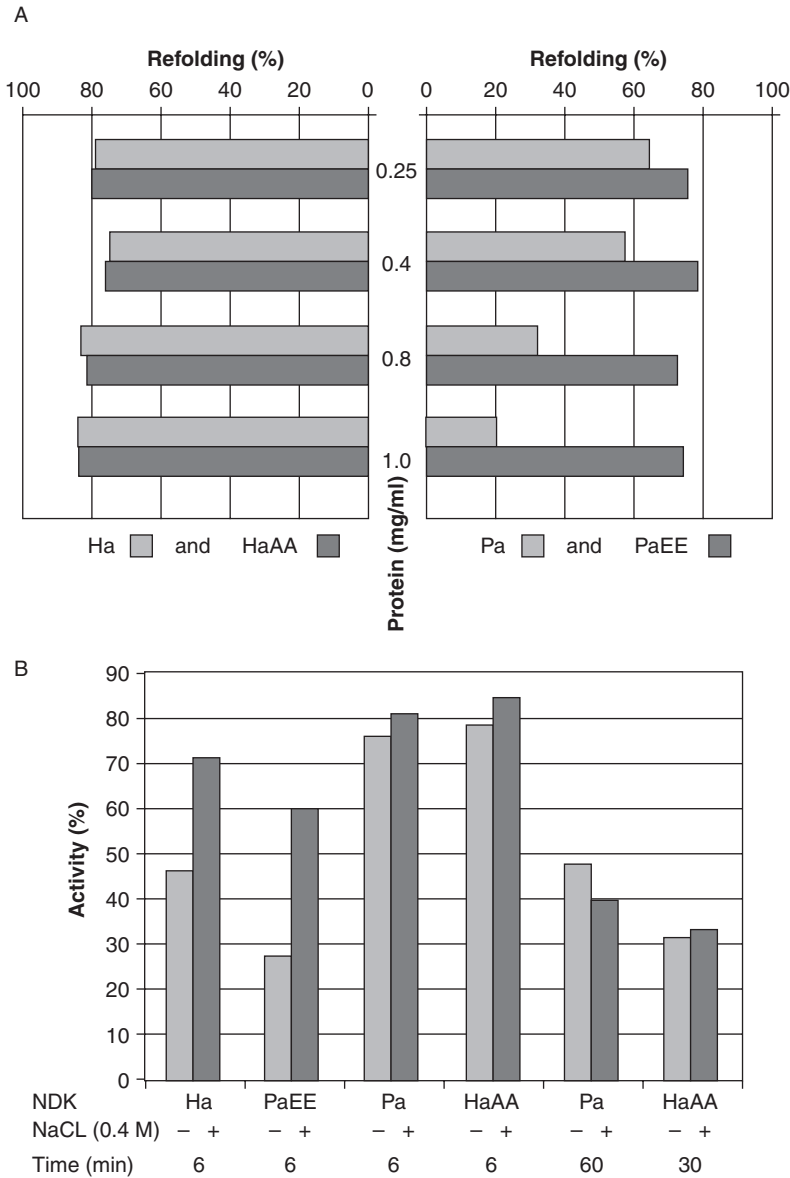


Figure 4.8. Stability of PaNDK and HaNDK and their mutants at different protein concentration. (A) Reversibility of thermal unfolding at different protein concentration. NDKs at protein concentrations shown were heat-treated at 85°C for 5 min, cooled for 30 min on ice, and then measured for enzyme activity. (B) Storage stability at dilute protein concentration. NDKs (0.5 μ g/mL) were incubated at 30°C for 6 to 60 min, in the presence (+) or absence (-) of 0.4 M NaCl. Enzyme activity immediately after dilution was taken as 100%.

With regard to stability, the wild-type HaNDK/EE showed protein-concentration dependence, gradually inactivated at low protein concentrations. The addition of salt protected the HaNDK from dilution-induced inactivation of HaNDK/EE. Similarly, the PaNDK/EE mutant showed stability against dilution-induced inactivation in the presence of salt, while the HaNDK/AA mutant and the wild-type PaNDK/AA were more stable against dilution independent of the salt concentration. The salt dependence of dilution stability was examined upon prolonged incubation, as plotted in Figure 4.8B. When PaNDK/EE mutant was incubated at 30°C for 6 min, addition of 0.4 M NaCl enhanced activity recovery (second column), similar to the halophilic HaNDK (first column). Such salt dependence was not observed for the nonhalophilic PaNDK (third column) and the HaNDK/AA mutant (fourth column). Longer incubation (30 and 60 min) resulted in lower recovery but no salt dependence for nonhalophilic PaNDK and HaNDK mutant (last two columns). These observations indicate that NDK/EE, but not NDK/AA, shows a salt dependence of dilution-induced inactivation. In this regard, the salt dependence of stability correlates with the oligomeric assembly of HaNDK and PaNDK mutants.

4.6. SOLUBLE EXPRESSION OF RECOMBINANT PROTEINS

As described above, halophilic proteins are highly soluble in both low- and high-salt environments. Utilizing such high solubility, we are developing a novel soluble expression vector based on halophilic proteins. This is perhaps the first application of proteins from halophiles, probably even from extremophiles, that is not based on their unique catalytic functions under abnormal environmental conditions.

In this section we describe two novel expression technologies using the unique nature of moderately halophilic bacteria and their proteins, aiming at enhancing the solubility of the proteins to be expressed. One of the unique properties of the halophilic world is high reversibility of thermally unfolded halophilic proteins, as described earlier. We described such halophilic properties for NDKs from both extreme and moderate halophiles. However, these proteins have a relatively large molecular mass and are oligomers. We searched for smaller and monomeric halophilic proteins with similar properties. Among several proteins cloned, halophilic β -lactamase (BLA) was highly soluble in both the native and thermally unfolded states. Such properties should allow a reversible refolding with high efficiency, which makes BLA an ideal fusion partner for soluble expression of aggregation-prone target proteins. The rationale for this is that when expressed as a fusion to BLA, high solubility of the unfolded BLA may help the target proteins maintain solubility in the nascent state and the reversible folding of the BLA portion and its high solubility may allow sufficient time for the target protein to fold. The second unique property of halophilic bacteria is accumulation of compatible solutes in the cytoplasm. As the compatible solutes enhance protein folding and stability (Yancey et al., 1982; Arakawa and Timasheff, 1983, 1984, 1985; Baskakov and Bolen, 1998; Baskakov et al., 1999; Uversky et al., 2001; Yancey, 2005; Street et al., 2010), one might utilize halophilic bacteria for cytoplasmic soluble expression of recombinant proteins. There are numerous examples in which compatible solutes increase the yield of in vitro protein refolding and the thermal stability of native proteins. However, attempts to express recombinant proteins using halophilic bacteria as a host are limited.

Techniques for efficient expression of native recombinant proteins become more and more important with recent advances in genomic and proteomic studies (Terpe, 2006;

Chou, 2007; Sahdev et al., 2008). These proteins are versatile tools for drug discovery, structure–function analysis, and biochemical analysis and are also often developed as therapeutic proteins by themselves. The most conventional system for heterologous expression of recombinant proteins is based on *E. coli*, which often leads to insoluble foreign proteins (called inclusion bodies), a major bottleneck for the current systematic studies of protein characterization and production. Many of the biochemically interesting families of mammalian proteins, including kinases, phosphatases, and other intra- and extracellular enzymes, are extremely difficult to produce as soluble proteins in *E. coli* cytoplasm (Esposito and Chatterjee, 2006).

Alternative to the direct expression in *E. coli* of heterologous proteins are the protein fusion techniques, a powerful method for the expression of foreign proteins in this host cell (Waugh, 2005; Arnau et al., 2006; Esposito and Chatterjee, 2006). One of the important goals in using fusion proteins is their ability for detection and purification using specific binding proteins to the partner protein. More important, the fusion partner may increase the solubility and folding efficiency of target proteins in fusion constructs (Kapust and Waugh, 1999; Zhang et al., 2004). High aqueous solubility and folding efficiency of halophilic proteins have attracted us to developing novel vector for expression of soluble proteins. We first selected BLA, a typical halophilic enzyme expressed in periplasm, as a candidate fusion partner. This enzyme, although a periplasmic protein, can be readily expressed as a native, active form in *E. coli* cytoplasm. Similar to many other halophilic enzymes (e.g., HsNDK, HaNDK, α -amylase from *Kocuria varians*, and its starch-binding domain), BLA is soluble upon denaturation by heat or urea treatments and hence can be refolded efficiently. Such high solubility and refolding efficiency make BLA an ideal fusion partner for soluble expression of aggregation-prone heterologous proteins in *E. coli*. An expression vector of the BLA-fusion protein, pBF, was constructed as shown in Figure 4.9 (Tokunaga et al., 2010b). The fusion protein vector consists of an N-terminal hexa-His-tag, a mature form of the halophilic BLA protein, followed by a thrombin cleavage site and a target protein. The gene encoding the target protein can be inserted into the vector at a multicloning site such as *SmaI*–*KpnI*–*SpeI*. High-level protein expression was directed by the T7 promoter and terminator. Using this BLA-fusion vector, we have succeeded in the soluble expression of human interleukin 1 α (IL1 α) with native biological activity. When expressed as a mature

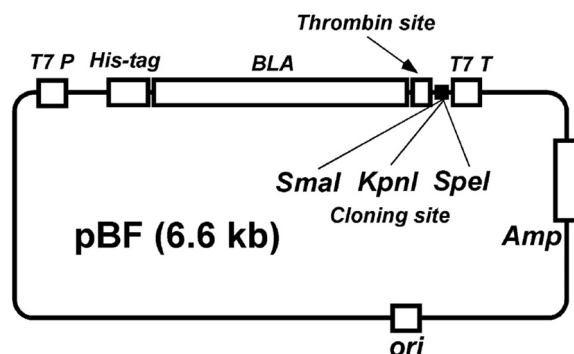


Figure 4.9. Construct of BLA-fusion vector. T7 P, T7 promoter region; T7 T, T7 terminator region; BLA, coding region of mature halophilic β -lactamase; Amp, ampicillin-resistant marker; ori, replication origin of plasmid. The backbone of this expression vector is pET15b (Novagen).

form, IL1 α , often forms inclusion bodies. Its refolding was found to be rather difficult. It is thus clear that fusion to BLA leads to suppression of aggregation of IL1 α moiety and spontaneous folding while on the fusion protein. IL1 α expressed with this vector was readily cleaved off from its fusion partner by thrombin digestion, generating a mature form of IL1 α with full biological activity.

The α -amylase gene isolated from a moderate halophile that grows in soy sauce mash exhibits an extremely high GC content, 71.4%. The expression of this gene product (mature form without secretion signal) with N-terminal His-tag in *E. coli* resulted in low recovery even when driven by a strong T7 promoter. When this protein was expressed as a BLA-fusion protein, however, its expression as a soluble protein increased at least two-fold over direct expression in *E. coli*. A small peptide, human neutrophil α -defensin (HNP-1), consisting of 30 amino acid residues, was expressed successfully as a BLA-fusion protein. It is a general consensus that expression of short peptides as a soluble form is difficult using an *E. coli* expression system. The mature HNP-1 was generated by digestion of fusion protein and showed bacteriocidal activity. Starch-binding domain from *Bacillus* sp. 195 α -amylase (BaSBD) forms inclusion bodies in an *E. coli* expression system. In contrast, BLA-BaSBD fusion protein was readily expressed in the soluble cytoplasmic fraction of *E. coli*. BaSBD in the fusion form and processed by thrombin digestion showed biological activity: binding to raw starch.

Moderately halophilic bacteria can grow over a wide range of salinities (0.2 M ~ saturated salts), optimally at 0.5 to 2.5 M salts. They accumulate small organic compounds called compatible solutes in the cells (Ventosa et al., 1998). Thus, cytoplasmic environments of halophilic bacteria are significantly different from those of *E. coli* and hence may provide an opportunity for the soluble expression of recombinant proteins. Compatible solutes, also called osmolytes, enhance the stability of the proteins and hence may increase the folding efficiency and/or protect proteins from degradation (Galinski, 1995). Host-vector systems to manipulate gene expression in this important bacterial group have been developed (Afendra et al., 2004; Vargas and Nieto, 2004). Several heterologous proteins were expressed successfully as a soluble protein in recombinant halophilic bacterial cells. Ice nucleation protein (InaZ) from *Pseudomonas syringae* (Arvanitis et al., 1995), green fluorescent protein from the jellyfish *Aequorea victoria* (Douka et al., 2001), and α -amylase from the hyperthermophilic archaeon *Pyrococcus woesei* (Frillingos et al., 2000) were reported to be expressed in *Chromohalobacter salexigens* and *Halomonas elongata*. We have recently succeeded in soluble expression in *Chromohalobacter* cells of a native nucleoside diphosphate kinase derived from extremely halophilic archaea (Nagayoshi et al., 2006) and human brain serine racemase (hSR) (Nagayoshi et al., 2009). To our knowledge, hSR is the first human protein expressed in moderate halophiles, meaning that their cytoplasmic environments are suitable for normal (nonhalophilic) and even human proteins as well.

We have developed strong promoters for the expression of heterologous proteins in moderate halophiles (Nagayoshi et al., 2006, 2009). We cloned the promoter regions of *hopP* (halophilic outer membrane porin) gene and chaperonin *groE* gene using halophilic β -lactamase as a reporter of promoter activity. We also developed a chloramphenicol-resistant determinant as a selection marker in *Chromohalobacter salexigens* (Nagayoshi et al., 2009). Thus, the recombinant expression in moderately halophilic bacteria was proved to be a promising candidate for producing soluble proteins in biotechnology applications. However, this system is still in the process of development and optimization and needs to be improved in several areas, including as an appropriate selection marker for the

transformants (e.g., an auxotrophic marker), as a tightly regulated inducible promoter, and in the preparation of competent cells for efficient transformation.

4.7. NATIVELY UNFOLDED PROTEINS

A large number of proteins and protein domains have been shown to have no discernible ordered structures under physiological conditions and hence are termed *natively unfolded* (Gast et al., 1995; Uversky et al., 2000; Chouard, 2011). Halophilic proteins share some characteristics with natively unfolded proteins: a low hydrophobic amino acid residue content and a high proportion of polar amino acid residues, in particular acidic amino acids. These characteristics render the natively unfolded proteins soluble, in contrast to the strong tendency of normal proteins to aggregate in the unfolded state. These characteristics also make the natively unfolded proteins difficult to fold, similar to extreme-halophilic proteins, under physiological conditions. The folding of extreme-halophilic proteins requires shielding of electrostatic charges and enhancement of hydrophobic interactions, which are provided by high salt concentrations. Even under such condition, the folding is slow (see Fig. 4.1). The enhanced folding by salting-out conditions is reminiscent of folding of α -synuclein, one of the classical natively unfolded proteins (Weinreb et al., 1996; Maiti et al., 2004; Yoon et al., 2008), by a folding enhancing osmolyte, TMAO (Baskakov et al., 1999; Uversky et al., 2001). This protein has been shown as a typical natively unfolded protein, characterized by excess acidic amino acids residues (Uversky et al., 2000). Recently, the “native” α -synuclein was shown to be an α -helical tetramer (Bartels et al., 2011; Wang et al., 2011), suggesting that the natively unfolded proteins are, in fact, folded under certain solvent or process conditions. Folding of halophilic proteins also depends on conditions. Once unfolded, it behaves as if it is a stable unfolded protein in aqueous solution, but under optimal salt conditions can fold into the native structure and often retains the native structure even after the removal of salt. Thus, it appears that natively unfolded proteins depend on the context of conditions surrounding the proteins.

NDK from extreme halophiles showed a unique salt-dependent folding pattern: Refolding was extremely slow in low-salt solutions and accelerated at higher salt concentrations. A tandem starch-binding domain of α -amylase from a moderate halophilic bacterium showed a similar behavior. Refolding was reversible in both cases (i.e., at low and high salt concentrations) and probably so for other halophilic proteins. Such slow, reversible folding of halophilic proteins should make them ideal for detailed structure analysis of folding intermediates. In particular, structure analysis of folding intermediates of oligomeric NDK may shed light on the relation between folding and self-association.

4.8. ORGANIC SOLVENT TOLERANCE

Many halophilic enzymes show varying degrees of tolerance to organic solvents (Marhuenda-Egea and Bonete, 2002; Fukushima et al., 2005; Karbalaeei-Heidari et al., 2007; Shafiei et al., 2010). They were more stable in nonmiscible than in water-miscible organic solvents (Shafiei et al., 2011). This is reminiscent of dried enzymes in organic solvents (Zaks and Klibanov, 1984, 1985, 1988; Volkin et al., 1991; Vermue and Tramper, 1995). It has been shown that certain enzymes, when freeze-dried and reconstituted with

organic solvents, show enzyme activity, although at considerably reduced catalytic activity levels (Zaks and Klibanov 1984). The activity observed was ascribed to dispersion, not solubilization, of the dry proteins, leading to sufficient exposure to the substrate of enzymes dissolved in organic solvents. The dispersed enzymes were more stable in nonmiscible organic solvents than in water-miscible organic solvents. Is there any relation between halophilic proteins and dry proteins in the presence of organic solvents? It appears that no mechanistic studies have yet been done to answer this question. Nevertheless, with regard to dry protein, high stability appears to be due to repulsion of the proteins from solvents, perhaps stronger repulsion for nonmiscible organic solvents. As nonmiscible organic solvents are highly unlikely to solvate the hydrophilic surface of native protein, a protein may form its own phase, separated from the solvent; the solvents simply supply substrates to and remove reaction products from the dry enzymes. When proteins unfold, however, both water-miscible and nonmiscible organic solvents may be able to solvate the protein. Thus, they may destabilize the proteins by solvating the unfolded structures. However, there may be a high-energy barrier to phase-separated proteins unfolding in nonmiscible organic solvents. The analogy may hold true for halophilic proteins in organic solvents. It is highly unlikely that organic solvents can solvate the surface of the native protein with excessive negative charges. As is the case for dry proteins, there may be a strong repulsion between organic solvents and the highly charged hydrophilic surface of halophilic proteins, leading to separation of the proteins from the solvent phase and a high-energy barrier to their unfolding. Once unfolded, organic solvents may solvate the unfolded protein to a greater degree than the native protein. Thus, both dry and halophilic proteins may be stabilized kinetically due to phase separation from organic solvents—more so than from nonmiscible organic solvents.

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FEATURES AND APPLICATIONS OF HALOPHILIC ARCHAEA

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5.1. INTRODUCTION

Halophilic organisms are organisms that are able to live in saline environments, being the presence of salt (generally, NaCl) a specific requisite for their growth. It is possible to find these types of organisms in all three domains of life: Bacteria, Eukarya, and Archaea (Litchfield, 1998). In particular, some of the most extreme halophilic microorganisms to date have been found inside the Archaea domain, requiring concentrations of NaCl next to saturation (between 3 and 5 M NaCl) for optimal growth. These haloarchaea live in hypersaline environments in which the concentration of salt exceeds that of seawater. Examples of these hypersaline ecosystems are salterns, hypersaline lakes, and evaporitic water bodies such as the crystallizer ponds of solar salterns (Oren, 1999a, 2002). These microorganisms have also been found in evaporitic deposits millions of years old (Fendrihan et al., 2006).

In addition to the natural environment, halophiles have also been isolated in molar concentrations from highly salty foods such as preserves, fermented products, and other foods preserved by salt treatment (Harrison and Kennedy, 1922; Thongthai et al., 1992; Namwong et al., 2011; Tapingkae et al., 2008). Most of the haloarchaea are orange, red, or purple, due to the presence of pigments in their cellular membranes, which are isoprenoid derivatives (Oren and Rodríguez-Valera, 2001).

Additionally, several physiological characteristics make haloarchaea especially well adapted to live in such hypersaline conditions (Litchfield, 1998). Perhaps the most relevant are the particular osmotic mechanisms that these microorganisms possess, which allow them to keep high intracellular ionic concentrations, and as a consequence of this fact, their special physiological features, including enzymatic machinery, are able to function under these conditions (Oren, 1999a,b). The haloarchaea are much better adapted to live in these hypersaline environments, as these high salt concentrations are generally an indispensable requisite for their cell integrity and viability (Soppa, 2006).

Phylogenetically, halophilic microorganisms in the Archaea domain can be found in the phylum Euryarchaeota, order Halobacteriales, and family Halobacteriaceae (Grant and Larsen, 1989; Grant et al., 2001). Although it is also possible to find halophiles in the families Methanospirillaceae and Methanosarcinaceae, halophiles are not the main organisms in these two families of methanogenic archaea.

At present (2011), the family Halobacteriaceae is divided into 28 accepted genera and includes more than 60 species (<http://www.the-icsp.org/taxa/halobacterlist.htm>). However, as Oren and Ventosa noted several years ago (2002), many more new species and genera appear in the literature or databases that need to be approved as new species or as representatives of recognized taxa. A complete list of haloarchaeal species can be found in the National Center for Biotechnology Information taxonomy database (<http://www.ncbi.nlm.nih.gov/>). In this chapter we focus on the family Halobacteriaceae, describing some of their most relevant aspects as well as exploring their current or potential biotechnological applications.

5.2. GENERAL FEATURES

5.2.1. Morphology

The members of the family Halobacteriaceae have certain peculiarities. One is related to their morphology, which can vary between cocci and rods but that can also include a variety of morphological types, thanks to the extreme pleomorphism of their cells. A variety of shapes—square flat cells, triangular cells, and trapezoidal cells—are seen. Variations in morphology can also occur due to environmental conditions, such as salt concentration.

It has been shown that the cell wall, which is the responsible for maintaining the cell shape, can vary in composition according to the genus. The noncocoid members of Halobacteriaceae have an S-layer cell wall composed primarily of glycoproteins. This type of wall requires a high NaCl concentration to maintain its integrity; it is destabilized in distilled water and in the absence of salt, the cell wall disintegrates and cell lyses.

On the other hand, the coccoid representatives possess a thick cell wall composed of a sulfated heteropolysaccharide as in the genus *Halococcus* (Steensland and Larsen, 1971) or of a poly(L-glutamine) glycoconjugate as in *Natronococcus* species (Niemetz et al., 1997). Contrary to the glycoprotein cell wall type, they do not require high salt concentration to maintain their integrity.

It has been shown that several species also require a high magnesium concentration (20 to 50 mM) or the presence of other bivalent cations to maintain the structural stability of the cell wall and its morphology (Cohen et al., 1983; Tindall et al., 1984). Some other adaptations to high-salinity conditions have been found as oxygen becomes a limiting factor in salt-saturated environments. The capacity of motility is one interesting adaptation, seemingly controlled by two different mechanisms: flagella and gas vesicles (Simon, 1981;

Alam and Oesterhelt, 1984). These structures, although not present in several species, can be advantageous since oxygen is generally more abundant at the surface of hypersaline water bodies (Beard et al., 1997).

5.2.2. Metabolism

The members of Halobacteriaceae are mainly aerobic heterotrophs, although some of their representatives are also able to grow anaerobically (facultative anaerobes). Aerobic respiration is dependent on the tricarboxylic acid cycle, which can be active alone or in parallel with the glyoxylate cycle, some reactions of the Embden–Meyerhof pathway, or with a modified Entner–Doudoroff pathway (Aitken and Brown, 1969; Serrano et al., 1998; Oren, 2006). The respiratory electron transport chain involves *b*-type cytochromes and low levels of other cytochromes (Cheah, 1970; Hallberg and Baltscheffsky, 1979; Hallberg-Gradin and Colmsjö, 1989; Sreeramulu et al., 1998; Tanaka et al., 2002).

The nutritional demands of haloarchaea vary widely according to species. Several haloarchaea do not grow on carbohydrates, requiring high concentrations of yeast extract and amino acids. Sometimes the presence of vitamins and nucleosides is also necessary (Dundas et al., 1963; Shand and Perez, 1999). Nevertheless, several members of the order Halobacteriales can grow using simple carbon sources such as acetate, glucose, or glycerol (Tomlinson and Hochstein, 1972, 1976; Vreeland et al., 2002). Moreover, in several species acetate is excreted when grown on glucose, which can be used in turn as a growth substrate. This mechanism was found in species such as *Haloferax volcanii*, *Halorubrum saccharovororum*, and *Halococcus saccharolyticus* (Bräsen and Schönheit, 2003).

In their natural habitats, many of these nutrients are excreted by other microorganisms which are inhabitants of hypersaline ecosystems. For example, many species can use glycerol, which is produced by the halophilic algae *Dunaliella salina*, as a carbon source (Borowitzka, 1981; Zvyagintseva et al., 1995). A few halophilic archaea can even grow on more complex sugars, such as glucose, galactose, lactose, fructose, or sucrose, but their oxidation is sometimes incomplete. This leads to the production of acids: acetate, pyruvate, and aldonic acid, among others (Hochstein et al., 1976; Tomlinson et al., 1978; Altekhar and Rangaswamy, 1992). In some species the addition of carbohydrates can be stimulating for growth (Gochner and Kushner, 1969).

Anaerobic respiration sometimes appears in facultative anaerobes as another adaptation to brines, since oxygen can become a limiting factor, due to its low solubility at high salt concentrations. This can be carried out using electron acceptors such as acetate, fumarate, nitrate, dimethyl sulfoxide, and trimethylamine *N*-oxide (Mancinelli and Hochstein, 1986; Oren and Trüpper, 1990; Oren, 1991; Kevbrina and Plakunov, 1992). Fermentation of L-arginine was also shown in the genus *Halobacterium* (Hartmann et al., 1980). Alternatively, some species (e.g., *Halobacterium salinarum*) can grow using a light-driven process, due to the presence of the bacteriorhodopsin proton pump. Bacteriorhodopsin is a protein that contains a retinal group, which is photosensitive. In this way, bacteriorhodopsin captures light energy, which is used to translocate protons across the outer membrane of the cell. As a consequence, a proton gradient is produced, which can then be used to generate chemical energy (ATP) (Oesterhelt and Stoekenius, 1971, 1973; Hartmann et al., 1980). Additionally, some species are able to grow anaerobically using inorganic nitrogen sources, such as nitrate, nitrite, or ammonium (Hochstein and Tomlinson, 1985; Mancinelli and Hochstein, 1986; Tomlinson et al., 1986; Tindall et al., 1989; Hochstein, 1991; Bonete et al., 2008).

5.2.3. Osmoadaptation

Unlike other halophilic microorganisms, which have osmoadaptation mechanisms based on the accumulation of compatible organic solutes (the Eukarya and Bacteria domains, with some exceptions), the osmotic adaptation mechanism in haloarchaea is based on the accumulation of high intracellular levels of K^+ (a strategy sometimes known as “salt in”). Instead of using organic solutes to counterbalance the high osmotic pressure of the hypersaline habitats in which they live, haloarchaea accumulate high concentrations of ions, mainly K^+ (but also Na^+ and Cl^- in minor proportions). The K^+ ions are pumped from the extracellular medium into the cytoplasm, through an Na^+/H^+ antiport, until the concentrations of K^+ ions nears 5 M in some cases reaching up to 6 M are reached (Christian and Waltho, 1962; Ginzburg et al., 1970; Lanyi and Silverman, 1972; Matheson et al., 1976). According to this fact, the entire intracellular machinery is necessarily adapted to the presence of high salt concentrations (Oren, 1999 a,b). In particular, the haloarchaeal enzymes show a special adaptation based on their amino acidic composition, having a predominance of negatively charged amino acids which are found mostly on the outer surface of the proteins. This is the result of a high content of acidic amino acids such as glutamate and aspartate (Reistad, 1970; Litchfield, 1998; Fendrihan et al., 2006). This negative charge surface makes it possible to maintain the protein conformation, stability, and activity when cations such as K^+ are present in the cytoplasm (Christian and Waltho, 1962; Mevarech et al., 2000; Britton et al., 2006; Oren, 2008).

However, it has been shown that several haloalkaliphilic species, including the genera *Natrialba*, *Natronobacterium*, and *Natronococcus*, are also capable of synthesizing organic solutes (2-glycine betaine or sulfotrehalose), which could partially replace the intracellular KCl. It is suggested that these osmoprotectants are produced only under special conditions, such as low nutrient availability (Desmarais et al., 1997; Grant, 2004).

Other divalent cations, such as Mn^{2+} and Mg^{2+} , are present in the cells of many haloarchaea, and it has been suggested that they may play a role in the mechanisms of osmotic adaptation because a correlation has been shown between the increased Mn^{2+} and Mg^{2+} concentration and the halophilic character of some bacteria and archaea (De Medicis et al., 1986).

5.2.4. Natural Habitats

It is common to find changes in the levels of salinity of brine lakes due to the equilibrium between the processes of evaporation and precipitation in the natural environments that these microorganisms inhabit. It is also well known that hypersaline environments may vary widely in their ionic composition according to the surrounding geology and also in relation to the climatic conditions (Grant, 2004). Several mechanisms make it possible for halophilic archaea to adapt to any variations in the conditions of salinity in the environment and to thrive despite somewhat harsh conditions in these environments (Soppa, 2006).

Variations in the composition of hypersaline water bodies and salt flats, among others, can have ecological relevance as well. To understand this, we first note that hypersaline environments can be classified into two main types: thalassohalines and athalassohalines. The former originated from the evaporation of seawater (from the Greek word *thalassa*, meaning “sea”). They have a composition that corresponds in general to that of seawater, with Na^+ as the principal cation and Cl^- as the principal anion. Their pH values range from

neutral to slightly alkaline, this being the case of the Great Salt Lake in Utah, among others (Grant, 2004). Several species have been isolated from that lake including *Halorhabdus utahensis* (Wainø et al., 2000).

The ionic composition in athalassohaline environments, however, differs greatly from that of seawater. The resulting pH values depend on the relation between divalent and monovalent cations. If the concentration of divalent cations is lower than that of monovalent cations, the pH values will be higher. This is the case with alkaline lakes such as the Magadi Lake in Kenya. If the concentration of the divalent cations is higher than that of the monovalent cations, saline water bodies will have a nearly neutral pH, as is the case of the Dead Sea in Jordan's Rift Valley (Grant, 2004). Examples of haloarchaea that inhabit these environments are *Natrialba magadii* and *Haloferax volcanii* (Mullakhanbai and Larsen, 1975; Tindall et al., 1984; Kamekura et al., 1997).

Accordingly, it is possible to find neutrophilic or alkaliphilic halophilic archaea inhabiting the foregoing environments that have physiological characteristics that allow them to adapt to these particular environments.

However, most of the literature refers to haloarchaea as microorganisms that grow at an optimum pH range of 6.0 to 10.0. Recently, Minegishi et al. (2008) found acidophilic haloarchaea restricted to grow only under acidic conditions. Their pH range for growth was determined to be 4.2 to 4.8, with an optimum pH of 4.4. Surprisingly, the strains were isolated from 28 different commercially available salts. However, these organisms are expected to be found inhabiting acidic natural environments. Acidic salty lakes are found in nature but are relatively scarce; most are in Western Australia and Chile. In these habitats the pH values vary between 1.5 and 4.0 (Benison et al., 2007; Benison, 2008). Studies performed in hypersaline lakes in Australia showed that the predominant ions in these habitats are Na^+ , Mg^{2+} , Cl^- , and SO_4^{2-} . It is supposed that the low pH values originate from the oxidation of sulfide and lignites in the subsurface. At the same time, the maintenance of acidic conditions could also be promoted by abiotic processes as ferrololysis and evapoconcentration, additionally including biotic processes promoted by microorganisms (Bowen et al., 2008).

The temperature ranges in the natural habitats in which haloarchaea can live are relatively wide. They were recognized as being mesophilic, thermotolerant, or moderately thermophilic within the range 30 to 50°C for optimal growth. However, species such as *Haloterrigena thermotolerans* can tolerate temperatures as high as 60°C (Montalvo-Rodríguez et al., 2000). Moreover, Ellis et al. (2008) have isolated halophilic archaea from hydrothermal vents from several locations, showing that they can even survive temperatures around 75°C for several minutes. The microbes were found to be related to the genus *Haloarcula*, which are also inhabitants of nongeothermal environments.

Haloarchaea can also tolerate low temperatures, as low as 4°C, a particularity that was seen in the antarctic strain *Halorubrum lacusprofundi* isolated from Deep Lake in Antarctica (Franzmann et al., 1988). However, their optimal growth rate is reached at 30 to 35°C. Based on several calculations, it has been speculated that, theoretically, two type strains of this species could survive temperatures as low as 2.4 and 1.1°C (McMeekin and Franzmann, 1988).

As well as surface hypersaline lakes and salt lakes formed by solar evaporation, important natural habitats for haloarchaea include other natural habitats, such as hypersaline soils, salterns, and "salt rocks" or evaporites. There are even ancient evaporitic deposits, whose ages range from 250 to 419 million years, from which several species of haloarchaea

have been isolated (Denner et al. 1994; Stan-Lotter et al., 2001; Grant, 2004; Gruber et al. 2004). Although these findings have highlighted the apparent longevity of these organisms in evaporitic salt rocks, it is still a matter of debate how the microorganisms remained viable in these deposits (Grant et al., 1998; Vreeland et al., 2000). Among the more recent findings are those dating from the Permian and the Triassic (Grant et al., 1998; McGenity et al., 2000; Radax et al., 2001; Vreeland et al., 2002; Mormile et al., 2003; Fendrihan et al., 2006). Moreover, the environmental conditions under which these organisms live—dry environments and high intensities of sunlight and temperature—make most of these cells especially well adapted to multiple types of environmental stresses (DasSarma and DasSarma, 2005; DasSarma, 2006).

It has been shown that some haloarchaea display features related to resistance to drying or even to vacuum (Kottemann et al., 2005; Abrevaya et al., 2011a). It has also been observed that organisms that are resistant to dehydration are also resistant to different temperatures (Coker et al., 2007) and to the effects of ionizing radiation (Crowe and Crowe, 1992; Kottemann et al., 2005; Whitehead et al., 2006; DeVeaux et al., 2007).

Because they are exposed to intense solar ultraviolet (UV) radiation, haloarchaea are also recognized as UV tolerant: adapted to various regimes and types of UV irradiation (Baliga et al., 2004; McCready et al., 2005; Abrevaya et al., 2009, 2011a). They have also shown resistance to transition metals (Zn, Cu, Ni, Co, Mn, and Fe) through an oxidative stress response (Kaur et al., 2006). It was also shown that the environment in which haloarchaea live also provides a natural shield that protects the microorganisms from the harmful effects of UV radiation due to the formation of halite crystals. The halite structure can absorb part of the radiation, attenuating its effect over the microorganisms (Fendrihan et al., 2009).

5.2.5. Pigments

With few exceptions, such as the pigmentless haloarchaea *Natrialba asiática* (Kamekura and Dyal-Smith, 1995), most halophilic archaea are characterized by their orange, red, or purple color, due to the presence of pigments on their cellular membrane (Oren et al., 1992; Oren and Dubinsky, 1994) (Fig. 5.1). These are isoprenoid derivatives such as retinal or carotenoids, mostly carotenoid linear chain derivatives such as α -bacterioruberin, among others (Kelly et al., 1970; Kushwaha et al., 1972, 1975; Rønekleiv et al., 1995). Carotenoids such as lycopene and β -carotene are less abundant (Kushwaha et al., 1972; Tindall, 1992).

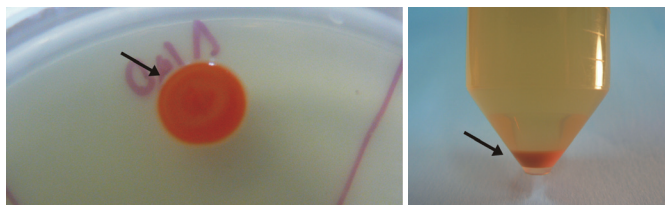


Figure 5.1. Pigmented cells in *Haloferax volcanii*. Left: spot on agar plate (arrow). Right: cells precipitated at the bottom of a liquid culture (arrow). (Courtesy of Dr. Ximena Abrevaya.) (See insert for color representation of the figure.)

In fact, the proton pump bacteriorrhodopsin, which is found in such species as *Halobacterium*, is dependent on the presence of a retinal pigment that is excited by the action of light. The activity of this protein allows these microorganisms to use light to obtain energy when nutrients are scarce or unavailable and oxygen concentrations are low (Brock and Petersen, 1976). A similar protein that makes use of retinal pigment is halorhodopsin, which works as a light-dependent chloride pump (Schobert and Lanyi, 1982), and sensory rhodopsins that play a role in phototaxis (Dencher and Hildebrand, 1979; Bogomolni and Spudich, 1982).

Because of the presence of these compounds, their colonies are orange or red pigmented. Moreover, sometimes the presence of dense communities of these microorganisms is responsible for a reddish color that it is possible to find in some of their habitats, such as hypersaline lakes (Fig. 5.2) (Oren and Dubinsky, 1994; Oren and Rodríguez-Valera, 2001). It has been shown that pigment production in these microorganisms is dependent on salinity, being lower at high salinities. The nutritional status of the cells appeared as another influencing factor for the pigment content (Gochner et al., 1972; Kushwaha and Kates, 1979; D'Souza et al., 1997).

Carotenoid pigments in haloarchaea have been related to shielding effects (Wynn-Williams et al., 2002), because of the fact that conjugate double bonds present in the



Figure 5.2. Salt lake in Namibia, Africa. The reddish colored water is due to the presence of halophilic-pigmented microorganisms, mostly haloarchaea. (Courtesy of Dr. Karsten Kotte, Institute of Earth Sciences, University of Heidelberg.) (See insert for color representation of the figure.)

pigments can be involved in the neutralization or elimination of their free radicals (Litchfield, 1998). Moreover, these pigments could participate in photoreactivation mechanisms, not only providing resistance to UV but also preventing damage related to ionizing radiation or hydrogen peroxide (Shahmohammadi et al., 1998).

5.2.6. Genetics

Taking in account the characteristics of the genome of haloarchaea, it was determined that DNA has a high G + C content, which makes it more stable in an intracellular environment that is exposed to high concentrations of cations (Litchfield, 1998). Many species have minichromosomes or megaplasmids, which in some cases can be as large as their chromosomes. These elements may be present in multiple copies per cell (Sapienza and Doolittle, 1982; Gutierrez et al., 1986; Ng et al., 1998). Plasmids could harbor important genes as the codificants for essential enzymes of metabolic pathways. The presence of extrachromosomal elements, which are highly variable and may constitute up to 30% of genetic material, has also been described (Litchfield, 1998; Macario et al., 1999; Brügger et al., 2002).

Additionally, poliploidy have been found in species such as *Haloferax volcanii*. Breuert et al. (2006) found that the chromosome copy number for *Haloferax volcanii* increases during the exponential phase (around 18 copies per cell) but decreases when the cells enter the stationary phase (10 copies per cell). The complete genome sequence of 12 haloarchaeal species has been obtained (DasSarma et al., 2010). The first complete sequence was obtained in 2000, corresponding to one of the most studied haloarchaea, *Halobacterium* sp. NRC-1.

5.3. APPLICATIONS OF HALOPHILIC ARCHAEA

Extremophilic organisms are the subject of numerous investigations, due to their strong resistance to diverse harsh conditions. These properties make them interesting not only in several basic research areas, but also in applied and biotechnological areas, including industrial applications. Nevertheless, despite their high potential from a biotechnological, industrial, and environmental point of view, in general, the potentialities and possible applications of members of the Halobacteriaceae family have not yet been fully explored and exploited. Notwithstanding, a number of applications have been proposed in a variety of biotechnological fields since they can be a source of valuable and unique compounds which are found only in these microorganisms. Several investigations have focused on the use of haloarchaea as a source of new enzymes that can be stable not only at high salt concentrations but also in organic solvents and other bioproducts, including biopolymers such as polysaccharides and biosurfactants. Technological uses have also been reported, with special emphasis on the haloarchaeal protein bacteriorhodopsin. Other uses are related to environmental applications such as bioremediation processes, wastewater treatment, and power generation in microbial fuel cells.

In fact, probably one of the first attempts to study haloarchaea came from the manufacture of salty preserved foods, since there are reports from the early twentieth century describing possible contamination with red-pigmented bacteria, many decades later identified as halophilic archaea. Moreover, the importance of haloarchaea in biotechnology and

in the industry is also related to the fact that the use of halophilic microorganisms presents some advantages over the use of nonhalophilic microorganisms.

These aspects are especially relevant at the industrial scale. Many of them are related to their particular conditions for growth. First, they can grow at high growth rates using simple substrates such as starch or acetate as carbon sources (Lillo and Rodríguez-Valera, 1990; Hezayen et al., 2000, 2001). Second, the contamination of cultures with other organisms can generally be prevented thanks to the high salt concentrations in their culture media. In this way the cultivation systems could be simplified to take advantage of new alternatives: for example, making use of open ponds (Hezayen et al., 2001). Third, it is easy to lyse the cells, simply by depletion of salts. This could be done by putting the cells in distilled water, providing a simple process for the recovery and purification of their products (Ventosa and Nieto, 1995; Hezayen et al., 2001).

Some drawbacks can also be associated with large-scale cultivation of haloarchaea, although there are no unsolvable problems. One is related to salts in their growth media, which could corrode the metal parts of bioreactors. This problem could be solved using corrosion-resistant reactors that have been specially developed for the growth of haloarchaea (Hezayen et al., 2000). Other problems could be associated with the availability of oxygen in aerobic cultures due to the fact that oxygen solubility depends on salt concentration. In this case, proper oxygenation for the cultures should be guaranteed (Shand and Perez, 1999). To date (2011) at least 158 patents have been published that make use of halophilic archaea or take advantage of their bioproducts. Furthermore, considering that hypersaline habitats are not excluded from the problem of environmental pollution and that some industrial processes generate saline wastewaters, additional environmental applications arise from the use of halophilic archaea.

Taking in account the interesting features of these microorganisms and the wide range of conditions they could tolerate, new applications emerge in scientific research since they could be used as a model for the study of the phenomenon of life on our planet and beyond, in such fields as astrobiology, exobiology, and evolutionary biology. Several industrial applications for haloarchaea are summarized in Table 5.1.

5.3.1. Production of Enzymes

Enzymes are of great importance as biocatalysts for various industrial and biotechnological applications. Some limitations arise because there are limiting conditions for their activity related to temperature range and pH, among others. In particular, enzymes isolated from extremophilic microorganisms, called extremozymes, have acquired great significance in recent times due to their stability under extreme environmental conditions being a new source of enzymes with new catalytic capabilities, activities, and applications.

Many haloarchaea are capable of producing extracellular and intracellular enzymes which are of biotechnological interest due to their functionality at high salt concentrations, and these enzymes are also stable under dry conditions. Additionally, many halophilic enzymes have other interesting properties, such as thermostability and the fact that, when isolated from haloalkalophilic organisms, they can work at high pH values. For this reason, enzymes isolated from haloarchaea are a main industrial target, among their other multiple applications.

TABLE 5.1. Examples of Potential Applications of Different Genera of Halophilic Archaea and Their Type Species

Genus	Examples of Species used in Biotechnology	Source of Isolation	Complete Genomic Sequence ^a	Examples of Potential Applications ^b
<i>Haloarcula</i>	<i>H. marismortui</i> <i>H. hispanica</i>	Dead Sea, Jordan Rift Valley Saltern, Spain	Yes No	Biosurfactant producer (*) Polysaccharide producer (*) Hydrocarbon-degrading (*) Salt-fermented food (*) Denitrifier, hydrocarbon degrading (<i>H. marismortui</i>) Ketocarotenoids (<i>H. hispanica</i>)
<i>Halobacterium</i>	<i>H. salinarum</i> <i>H. sp. NRC-1</i> <i>H. sodomense</i> <i>H. sp. GRB</i> <i>H. sp. SP1(1)</i>	Dead Sea, Jordan Rift Valley Salted food Dead Sea Jordan Rift Valley Saltern ponds of Gruissan, Mediterranean coast of France Salt pans in Kandla, India, and other sources.	No Yes No No No	Bacteriorhodopsin and halorhodopsin (*) Aldehyde dehydrogenase (*) Salt-fermented food (*) Halocines (*) Resistance to mercury and volatilization capabilities (*) Recombinant gas vesicles used for separation, antigen presentation and vaccine development (<i>H. sp. NRC-1</i>). Detoxifying enzyme for arsenite (<i>H. salinarum</i>) Amyloglucosidase (<i>H. sodomense</i>) Drug testing (GRB) <i>Halobacterium</i> sp. SP1(1) accelerated the fish sauce fermentation Extracellular protease resistant to organic solvents [<i>H. sp. SP1(1)</i>]
<i>Halobiforma</i>	<i>H. haloterrestriis</i>	Hypersaline soil close to Aswan, Egypt	No	PHA producer
<i>Halococcus</i>	<i>H. morrhuae</i> <i>H. saccharohyticus</i>	Dead Sea, Jordan Rift Valley Salterns in Spain	No	PHA producer (*) Resistance to mercury and volatilization capabilities (*) Siderophore producer (*)

<i>Haloferax</i>	<i>H. volcanii</i> <i>H. mediterranei</i> , <i>H. alicantei</i> <i>H. denitrificans</i> <i>H. alexandrinus</i>	Dead Sea, Jordan Rift Valley Salterns near Alicante, Spain Salterns in San Francisco Bay, CA Solar saltern in Alexandria, Egypt Solar salterns of Cabo Rojo, Puerto Rico	Yes No No No No	Resistance to mercury and volatilization capabilities (*) Hydrocarbon degrading (*) Halocines (sp.) α -galactosidase (<i>H. alicantei</i>) PHA producer (<i>H. mediterranei</i>) Exopolysaccharide producer (<i>H. mediterranei</i>) Canthaxanthin producer (<i>H. alexandrinus</i>) Siderophore producer (*)
<i>Halogeometricum</i>	<i>H. borinquense</i>		No	Siderophore producer (*)
<i>Haloquadratum</i>	<i>H. waslbyi</i>	Salt crust hypersaline pool, Sinai Peninsula	Yes	PHB producer
<i>Halorhabdus</i>	<i>H. utahensis</i>	Great Salt Lake, Utah	Yes	α -xylosidase
<i>Halorubrum</i>	<i>H. sodomense</i>	Dead Sea, Jordan Rift Valley	Yes	Carotenoid producer (<i>H. sodomense</i>); siderophore producer
<i>Haloterrigena</i>	<i>H. turkmenica</i>	Sulfate saline soil in Turkmenistan	Yes	Siderophore producer
<i>Halovivax</i>	<i>H. asiaticus</i>	Saline Lake Ejnor, Inner Mongolia, China	No	Biopolymer producer; biosurfactant producer
<i>Natrialba</i>	<i>N. aegyptica</i> , <i>N. magadii</i>	Magadi Lake, Kenya	Yes	Extracellular protease; siderophore producer; polyglutamic acid producer (<i>N. aegyptica</i>)
<i>Natronobacterium</i>	<i>N. gregoryi</i>	Magadi Lake, Kenya	No	PHA producer
<i>Natronococcus</i>	<i>N. occultus</i> <i>N. amylolyticus</i>	Magadi Lake, Kenya	No	Lipolytic activity (*)
<i>Natronomonas</i>	<i>N. pharaonis</i>	Highly saline soda lakes in Egypt and Kenya	Yes	α -Chymotrypsinogen

^aFull genome sequencing was completed for several species.

^bApplications marked with an asterisk are attributed to the genus.

As mentioned, the haloarchaeal proteins require high ionic strength to maintain their function and structure, due to the large amount of intracellular KCl that haloarchaea accumulate to cope with high salt concentrations (Dym et al., 1995; Madern et al., 2000).

Exoenzymes with degradative macromolecular properties, such as hydrolases, were found in haloarchaea such as proteases, amylases, amyloglucosidases, and lipases. Other active enzymes have also been found: β -galactosidase, β -xylanase, β -xylosidase, and nuclease (Ventosa and Nieto, 1995; Bonete and Martínez-Espinosa, 2011). Several of these enzymes can be active not only at high salt concentrations but also at high temperatures and alkaline pH values, varying in their NaCl concentration requirements (between 1 and 23% NaCl for optimal activity) (Ventosa et al., 2005).

Very few of these enzymes have been used in processes on an industrial scale. This is because there is little demand for manufacturing processes that require salt-tolerant enzymes; however, some of these extremozymes have begun to be exploited on a commercial scale (Eichler, 2001). Moreover, the ability of these enzymes to remain stable in environments with low water activity also allows them to be stable in the presence of high levels of organic solvents, which would lead to additional applications. Such is the case of the extracellular protease of *Halobacterium halobium*, which can act as a biocatalyst in aqueous and organic media (Kim and Dordick, 1997). The utility of these enzymes has been reviewed by Klivanov (2001). Other possible applications are related to their use as biocatalysts in organic solvents used in reverse micellar systems (Marhuenda-Egea and Bonete, 2002).

Other possible applications of halophilic proteases are related to their uses as detergent additives, such as is the case for B-chymotrypsinogen from *Natronomonas pharaonis*, which has activity at salt concentrations as low as 3 mM (Stan-Lotter et al., 1999). There are many industrial and biotechnological applications of halophilic proteases. More detailed characteristics of these enzymes and their applications can be found in papers by De Castro et al. (2005, 2008), D'Alessandro et al. (2007), and Akolkar and Desai (2010).

Other enzymes, such as α -amylase, were isolated from such species as *Natronococcus amylolyticus*, *Haloarcula* spp., *Haloferax mediterranei*, *Haloferax alicantei*, and *Halobacterium salinarum* (Rodríguez-Valera et al., 1983; Kobayashi et al., 1992; Kanai et al., 1995; Bagai and Madamwar, 1997; Pérez-Pomares et al., 2003). An amyloglucosidase of *Halorubrum sodomense* has also been characterized (Chaga et al., 1993), and production of enzymes such as β -xylanase and β -xylosidase was found in the extremely halophilic archaeon *Halorhabdus utahensis* (Wainø and Ingvorsen, 2003).

Enzymes with lipolytic activity are also important for various processes related to the pharmaceutical industry and the hydrolysis of fats, with environmental applications. It was possible to characterize a lipolytic activity in *Natronococcus* spp., an activity that had not been reported previously in the Archaea domain (Boutaiba et al., 2006). The ability to produce proteases and esterases has also been studied in the haloarchaeon *Haloarcula marismortui* (Camacho et al., 2009).

Halophilic nucleases from *Halococcus* species have also been found that have important applications for laboratory use as restriction enzymes (Obayashi et al., 1988). In addition to enzymes with hydrolitic activity, isomerases such as peptidyl prolyl *cis-trans* isomerase have also been found. The gene coding for this enzyme in *Halobacterium cutirubrum* has been patented by Iida et al. (1997) for the development and manufacture of novel immunosuppressant and physiologically active substances and for the production of recombinant proteins.

5.3.2. Production of Biopolymers

Biopolymers of microbiological origin are excellent candidates to replace synthetic polymers. It is possible to find a number of endo- and exopolymers synthesized by bacteria and halophilic archaea that are of importance for the biomaterials industry. One example is biodegradable plastic because of its applications in the oil industry due to its surfactant properties and its uses as an emulsifier that increases the efficiency of oil extraction processes, especially because oil reservoirs are often high-salinity-water reservoirs (Fernandez-Castillo et al., 1986; Rodríguez-Valera, 1992; Romano et al., 1996; Nicolaus et al., 1999b).

Polyhydroxyalkanoates. Polyhydroxyalkanoates (PHAs) are a family of biodegradable polymers of microbiological origin that are produced by bacterial fermentation of sugar or lipids and accumulated in many microorganisms as carbon and energy storage sources. Chemically, they are linear polyesters, the most common type of PHAs being poly(β -hydroxybutyrate) (PHB) and poly(β -hydroxyvalerate) or copolymers such as poly(β -hydroxybutyrate-co-3-hydroxyvalerate) (PHBV). These compounds are considered a source of biodegradable materials and plastics with broad applications and advantages over other bioplastics, such as poly(lactic acid), because of their UV stability (Koller et al., 2010). Although they were discovered in 1972 in the haloarchaeon *Haloarcula marismortui* (Kirk and Ginzburg, 1972), their use is limited due to high production costs.

Several strains of the Halobacteriaceae family, the genera *Haloferax*, *Halobiforma*, and *Haloquadratum* have been found to accumulate PHB. PHA was also detected in strains from the genera *Halorubrum*, *Haloarcula*, and *Halococcus* (Legat et al., 2010) (Fig. 5.3).

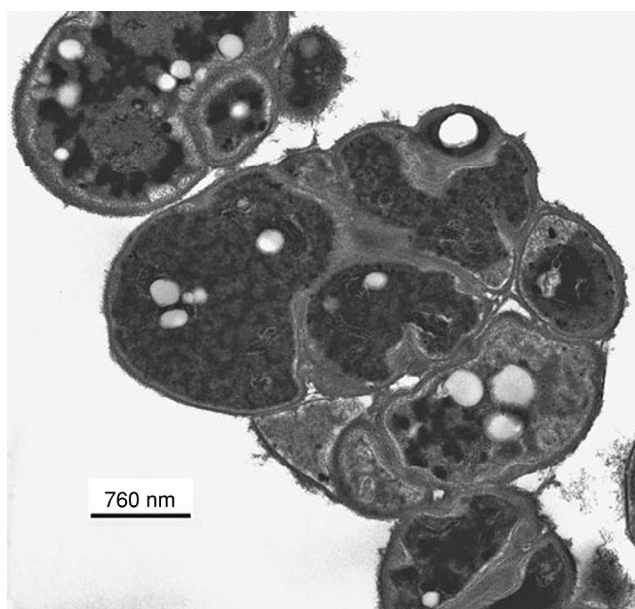


Figure 5.3. Transmission electron micrograph of *Halococcus morrhuae* DSM 1307T cells containing whitish poly(β -hydroxybutyrate) inclusion bodies. [From Legat et al. (2010), with permission from Springer Science + Business Media.]

In particular, *Haloferax mediterranei* was identified as the best haloarchaeal PHA producer, being capable of generating large amounts of PHB (around 60% dry weight), a copolymer of β -hydroxybutyrate and β -hydroxyvalerate (Rodríguez-Valera et al., 1991; Legat et al., 2010), depending on salinity, carbon source, and phosphate concentrations (Fernandez-Castillo et al., 1986; Lillo and Rodríguez-Valera, 1990; Rodríguez-Valera and Lillo, 1992).

The use of *Haloferax mediterranei* and other halophilic archaea offers advantages over industrial-scale PHA producers (e.g., as the bacteria *Ralstonia eutropha*), since they can be grown on low-cost carbon sources (Chen et al., 2006; Koller et al., 2007) and purification of the product is easy, as the cells can simply be lysed in water (Ventosa and Nieto, 1995). The extremely halophilic archaea strain 56 is capable of accumulating large amounts of PHB (around 0.43 g per gram of dried cells) (Hezayen et al., 2000, 2002).

Polysaccharides. Polysaccharides of microbial origin have a myriad of applications and biotechnological potential in fields such as medicine, agriculture, and the food industry. Moreover, it has been shown that they could also be used in processes related to wastewater treatment, as gelling and thickening agents, as emulsifiers, for viscosity stabilization, in oil recovery, and in the rheology of aqueous systems (Ventosa and Nieto, 1995; Nicolaus, 2004).

The Halobacteriaceae family appears to be an interesting source for use in polysaccharide production (Anton et al., 1988; Severina et al., 1988; Rodríguez-Valera et al., 1991). These biopolymers have generally been shown to be excreted into the extracellular medium in the form of exopolysaccharides. The natural role of these polysaccharides in these microorganisms could be related to a protection mechanism against environmental stresses in their habitats or acting as a barrier against the penetration of viruses (Hayshi et al., 1996; Riccio et al., 1996; Nicolaus et al., 1999a).

It has been shown that two genera in Halobacteriaceae are good producers of exopolysaccharides: *Haloferax* and *Haloarcula*, in particular the species *Haloferax mediterranei*, *Haloferax gibbonsii*, *Haloferax volcanii*, and *Haloferax denitrificans* and several *Haloarcula* strains (Anton et al., 1988; Severina et al., 1990; Parolis et al., 1996; Paramonov et al., 1998; Nicolaus et al., 1999a,b).

Some interesting properties, such as high viscosity at low concentrations, resistance to temperature, and pH, in addition to tolerance to high salt concentrations, have been identified in the sulfated exopolysaccharide isolated from *Haloferax mediterranei* strain R4, whose structure was elucidated (Parolis et al., 1996).

Another extremely halophilic archaeon, *Natrialba aegyptiaca* (*aegyptia*) strain 40, was found to excrete an extracellular polymer, composed primarily of glutamic acid (85% w/w), as poly(glutamic acid) (Hezayen et al., 2001). This could have multiple applications since it has been shown that poly(glutamic acid) has been used in hydrogels, as a thickener, as a humectant, as an emulsionant, as an additive and solubilizer, and as a drug carrier. It has also been proposed for water treatment and as a heavy metal sequestering agent, coagulant, or flocculant, and as an adhesive. It is also biodegradable.

Biosurfactants. Biosurfactants, surfactants of biological origin, are a structurally diverse group of molecules from a chemical point of view. Chemically, it is possible to classify them into surfactate particles, polymeric biosurfactants and glycolipids, lipopeptides and lipoproteins, phospholipids, neutral lipids, and fatty acids (Georgiou et al., 1992).

They are recognized as tensoactive agents, presenting high surfactant activity and emulsifying properties, as well as, low toxicity and biodegradability. They are produced by microorganisms, mainly prokaryotes (Maier, 2003). These properties make them useful in several biotechnological applications and in the bioremediation of contaminated environments. They can also be used as surfactants for oil recovery in the dispersion of oil leakage in both soils and waters, including enhanced oil recovery (Georgiou et al., 1992; Banat, 1995).

Other uses are related to the formulation of asphalt, cement, paints, wood preservatives, textile products, treatment of metals and water, mining, and as emulsifiers and moisturizers in food and cosmetic manufacture (Layman, 1985; Kosaric et al., 1987; Desai and Banat, 1997; Banat et al., 2000; Cameotra and Makkar, 2004). However, despite the amazing number of applications of these molecules, little has been done on the screening of halophilic archaea as biosurfactant producers (Cameotra and Makkar, 1998). The most recent work is that of Kebbouche-Gana et al. (2009), who identified two haloarchaea strains from an Algerian culture collection. These strains (A21 and D21) were shown to be optimal as biosurfactant producers, with a high emulsion-stabilizing capacity under extreme pH (from 2 to 11), NaCl and ethanol concentrations (up to 35% and 25% in the aqueous phase respectively). The analysis of its 16S rRNA gene sequences, showed that the two strains of Halobacteriaceae corresponds to the genera *Halovivax* (strain A21) and *Haloarcula* (strain D21). Membrane lipids from haloarchaea could be used as surfactants as Post and Al-Harjan have suggested (1988), in particular as surfactants for oil.

5.3.3. Uses in Fermented Foods

As noted earlier, perhaps the first studies on halophilic archaea were carried out on fermented foods with a high salt content, as there was a need to understand some problems associated with contamination and decomposition of these foods. Some of these works are documented in several historical papers (Klebahn, 1919; Harrison and Kennedy, 1922; Petter, 1931; Lochhead, 1934). The truth is that in order to be produced or preserved, fermented foods require the action of microorganisms. For foods with molar concentrations of salt, the participation of haloarchaea appeared to be essential.

Perhaps one of the more traditional seasonings of some cultures in Southeast and East Asia is fish sauce. This preparation, which is characteristically reddish brown, contains a high concentration of NaCl. The procedure involves the addition of salt and fish, which then undergo a long fermentation period (Yongsawatdigul et al., 2007). The high salt concentration of the sauce allows the growth of halophilic microorganisms (Tapingkae et al., 2008) which are believed to participate in the fermentation process (Thongthai and Siriwongpairat, 1978). The salt concentration in traditional fish sauce is very high, about 25% NaCl (Tungkawachara et al., 2003). Few members of the Halobacteriaceae are able to thrive in such extreme salt concentrations, and in this concentration range, haloarchaea are within their range for optimal growth (Grant et al., 2001). In fact, several strains of haloarchaea were isolated from fish sauce: *Halobacterium salinarum* and *Halococcus* sp., which reach their maximum densities after 3 or 4 weeks. Moreover, new species, such as *Natrinema gari*, have been isolated from fish sauce (Tapingkae et al., 2008), *Natronococcus jeotgali* and *Halalkalicoccus jeotgali* from other foods, such as shrimp *jeotgal* (Roh et al., 2007 a,b), and *Halobacterium piscisalsi*, and *Halorubrum cibi* from fermented fish (Yachai et al., 2008; Roh et al., 2009).

Additionally, some of these foods (e.g., fish sauce), as part of the process, are exposed directly to the sun, and as the recipes suggest, this helps to “digest the fish and turn them into fluid.” It is supposed that the digestion of the fish is possible due to enzymes with proteolytic activity that are involved in this process, an environment in which only haloarchaeal extremozymes can be active without denaturation (Thongthai and Siriwongpairat, 1990; Thongthai and Suntinalert, 1991; Thongthai et al., 1992; Vidyasagar et al., 2006). Several strains of *Halobacterium salinarum*, *Halobacterium* spp., and *Halogetometricum* spp. were identified as protease producers.

Recent studies tried to test the use of haloarchaeal strains as starter cultures in the production of traditional fermented foods, mimicking the natural fermentation processes with very good results not only in accelerating the process itself but also in improving their safety and quality (Akolkar et al., 2009; Aponte et al., 2010). Other applications of haloarchaea in the food industry are related to the use of some of their bioproducts as additives, among others (Post and Collins, 1982).

5.3.4. Uses in Bioremediation and Xenobiotic Degradation

The saline environments distributed widely on the planet, such as hypersaline waters and soils, are not excluded from the effects and consequences of pollution. At the same time, several industrial processes, such as the production of chemicals (herbicides, pesticides, pharmaceuticals), extraction of petroleum, leather industries, among others extraction are capable of generating hypersaline wastewater as final disposals (Oren, 1993; Lefebvre and Moletta, 2006).

It has been noted that approximately 5% of total world wastewater effluents are highly saline (Lefebvre et al., 2005). Petroleum extraction contributes to this figure; it was estimated that for every barrel of oil produced, 10 barrels of saline water (1 to 250 g/L) are generated (Cuadros-Orellana et al., 2006). Because of this, the use of halophilic microorganisms in the treatment of contaminated saline effluents and soils, hydrocarbon-polluted environments, and the degradation or conversion of toxic wastes due to their tolerance not only to salt but also to organic contaminants is of great interest (Schiraldi et al., 2002; Ding and Lai, 2010).

Through time, there was an increasing finding of representatives inside the family Halobacteriaceae showing developed catabolic abilities, although initially it was believed to have a restricted metabolic diversity (Oren, 2002). Moreover, several haloarchaeal strains showed good potential for their application in bioremediation processes.

Conventionally, contaminated materials are physicochemically treated because the more common alternative biological treatments are inhibited by salts (Pieper and Reineke, 2000; Oren, 2002). Alternative approaches using halophilic microorganisms appear to be a good solution, and the extreme halophilic archaea seems to be promissory to achieve this objective (Al-Mailem et al., 2010). Besides this, organic matter is usually present in wastewater disposals, and it has been shown that microorganisms can be part of the biological treatment of carbonaceous, nitrogenous, and phosphorous disposals at high salt concentrations (Lefebvre and Moletta, 2006). For example, haloarchaeal denitrifiers such as *Haloferax mediterranei* or *Haloarcula marismortui* could have an important role in the treatment of saline wastewaters contaminated with ammonia, nitrite, or nitrate (Martínez-Espinosa et al., 2007). A detoxifying enzyme for arsenite has been detected in *Halobacterium salinarum*,

which could have interesting applications in the bioremediation of this chemical pollutant in the environment (Wang et al., 2004). Additionally, haloarchaeal strains belonging to the genera *Haloferax*, *Halobacterium*, and *Halococcus* have shown mercury resistance and contribute to its volatilization, which could be combined usefully to remove toxic mercury compounds (Al-Mailem et al., 2011).

Furthermore, some authors described the capability of some strains to oxidize petroleum hydrocarbons (Bertrand et al., 1990; Kulichevskaya et al., 1992; Tapilatu et al., 2010). Most of these microorganisms were isolated from oil deposits from different environments around the world. Kulichevskaya et al. (1992), as well as other authors, described the capability of hydrocarbon-degrading haloarchaea to degrade a wide range of *n*-alkanes and polynuclear aromatic hydrocarbons at about 30% NaCl concentration. Some of these haloarchaea were identified as belonging to the genera *Haloarcula*, *Halobacterium*, and *Haloferax* (Emerson et al., 1994; Zvyagintseva et al., 1995; Oriel et al., 1997).

Through partial genome analysis it was also possible to identify genes related to putative xenobiotic-degrading enzymes possibly involved in the degradation of several compounds, such as 1,2-dichloroethane, naphthalene and anthracene, γ -hexachlorocyclohexane, 1- and 2-methylnaphthalene, and benzoate in haloarchaea (Ding and Lai, 2010). *Haloferax* sp. D1227 was also capable of growing on aromatic compounds, (Emerson et al., 1994). The degradation of other pollutant compounds, such as halogenated hydrocarbons, including trichlorophenols and insecticides such as DDT, were also documented (Oesterhelt et al., 1998). However, there is still much work to be done to increase the information related to the role of halophilic archaea in bioremediation processes (Le Borgne et al., 2008).

5.3.5. Uses in Solar Salt Production

Another possible use of halophilic archaea is related to salt production (Davis, 1974; Jones et al., 1981; Javor, 2002). The existence of dense communities of these microorganisms in natural saltern crystallizer ponds or in human-made solar salterns can contribute to the process of obtaining salt, physically or chemically. In the first case they can influence the evaporation process, increasing the temperature of water, thus increasing the evaporation rate and contributing to the process of salt production. The presence of pigments in haloarchaea such as carotenoids has an essential role in the absorption of radiation from sunlight. In the second case they can excrete products that could chemically affect salt precipitation processes.

Several cultivable species from the Halobacteriaceae family have been isolated from various solar salterns belonging to the genera *Haloferax*, *Halorubrum*, *Haloarcula*, *Halobacterium*, *Halogeometricum*, *Halosarcina*, *Halorubrum*, *Haloquadratum*, and *Natrinema*, (Asha et al., 2005; Pašić et al., 2005; Manikandan et al., 2009; Cui et al., 2011; Trigui et al., 2011). However, different halophilic or halotolerant microbial communities are present in solar salterns and also take part in this process (Oren et al., 2009) since these habitats showed diverse groups of microorganisms (Manikandan et al., 2009), although the dominant group appears to be halophilic archaea (Pašić et al., 2005). Moreover, some reports suggested a possible role of haloarchaea in evaporitic processes such as the crystallization of rocks of salt composed of NaCl (halite) (Castanier et al., 1999). Some of the

microbiological processes that drive crystallization in these solar salterns still need to be characterized (Javor, 2002).

5.3.6. Carotenoid Derivative Production and Biotechnological Uses

The pigments that haloarchaea produce are isoprenoid derivatives, mainly C50 bacterioruberin derivatives and other carotenoids, which are compounds that can be valuable commercially. This is the case of the keto-carotenoid derivative canthaxanthin, isolated from the new species *Haloferax alexandrinus* (Asker and Ohta, 1999, 2002; Margesin and Schinner, 2001). Moreover, Calo et al. (1995) measured the carotenoid content of the haloarchaea *Halobacterium salinarium*, *Haloarcula hispanica*, and *Haloferax mediterranei* and showed that all contained high levels of several ketocarotenoids.

Applications of canthaxanthin are related to its use in animal foodstuffs, as a food additive, and in the cosmetic industry. Finally, other applications of these and other isoprenoid derivatives have been proposed, although never carried out in practice (Khanafari et al., 2010; Litchfield, 2011).

The influence of nutrients in carotenoid production has also been demonstrated (Fang et al., 2010), as has that of other factors affecting the expression of carotenoid pigments in pollutants such as aniline (Raghavan and Furtado, 2005).

Another isoprenoid derivative, the retinal pigment, is responsible for the light dependence of the proteins bacteriorhodopsin and halorhodopsin in haloarchaea. Both naturally occur as light-driven pumps in haloarchaea, but they have several interesting biotechnological applications, not only based on their properties as ion pumps but also properties related to the photochemical and photoelectrical features of these molecules, especially bacteriorhodopsin, for which many uses have been patented (Oesterhelt et al., 1991; Hampp, 2000a,b; Margesin and Schinner, 2001). Moreover, currently bacteriorhodopsin protein is commercially available (Munich Innovative Biomaterials, Germany; <http://www.mib-biotech.de>).

It is interesting to note that bacteriorhodopsin has some amazing properties since it is very stable even in the absence of salts, resisting the effects of sunlight and oxygen but also digestion by most proteases. It is active in a wide temperature range between 0 and 45°C but can also tolerate temperatures around 80°C in water and up to 140°C in a dry state. It is also active in the whole pH range from 1 to 11 (Chen and Birge, 1993). Many uses are associated with optoelectronics: for example, using bacteriorhodopsin in holography for image storage (Oesterhelt et al., 1991) or in bioelectronics for the production of computer memories with the benefit of a high density of storage and parallel processing (Oesterhelt et al., 1991; Birge, 1995; Birge et al., 1999).

Due to the fact that bacteriorhodopsin is a molecule that is easy to immobilize, bacteriorhodopsin films have been developed and used as light-sensitive photoelectric devices and as chemical sensors or biosensors, including ultrafast light-detection sensors and photosensitive chromoprotein films (Miyasaka, 1992; Koyama et al., 1994; Ackley and Shieh, 1998; Margesin and Schinner, 2001). Other potential uses include its property as an energy converter from sunlight to electricity (Skulachev, 1976; Ang and Sammells, 1980), as an ATPase pH-sensitive dye (Minoru et al., 1992), and as a biophotochemical reactor for hydrogen production (Sediroglu et al., 1999). Halorhodopsin, the haloarchaeal chloride pump, has been proposed as a light-sensitive chloride-based biosensor (Seki et al., 1994).

5.3.7. Other Applications

Some genetically engineered vectors have been constructed (Miao et al., 2009) that make use of specific genes from haloarchaea species. These include genes encoding proteins necessary for the synthesis of gas vesicles (DasSarma et al., 1999; Stuart et al., 2001), genes that share common epitopes with the human c-myc oncogene protein from *Halobacterium salinarum* (Ben-Mahrez et al., 1988, 1991), and genes related to the expression of other human membrane proteins (Sölemann et al., 1997).

Additionally, haloarchaeal cells or plasmids such as pGRB-1 (from *Halobacterium* strain GRB-1) (Ebert et al., 1984) were proposed for their use in the testing of drugs in a prescreening step in vivo or in vitro, respectively, in particular for drugs that interfere with DNA, as antitumor drugs or antibiotics, since haloarchaea appears to show sensitivity to some eukaryotic-type drugs. The plasmid pGRB-1 offers a simple method for visualization of the damage by simple electrophoresis of DNA being possible to see fragments as a product of DNA-induced breakage or other effects reflected in changes in the mobility of the plasmid (Sioud et al., 1987; Forterre, 1989; Forterre et al., 1991).

Ether lipids from haloarchaea have potential applications in the form of new types of liposomes, in contrast with the common ester-lipid liposomes, since they are more resistant to pH, temperature, and chemical and enzymatic degradation, including biodegradation from other microorganisms (Galinski and Tindall, 1992). These liposomes were proposed for using as deliverers of drugs and even vaccines (Patel et al., 2002; Omri et al., 2003).

Halocins are peptides produced by halophilic archaea (mostly haloarchaeal rods) that showed inhibitory activity directed to halophages or archaea (Rodríguez-Valera et al., 1982). These proteins have shown proteolytic activity (some of them identified as serine proteases) and exhibited very high stability, with respect to temperature and pH. Not all of these proteins displayed salt dependence. Some of these characterized halocines were isolated from *Halobacterium*, *Haloferax*, and other haloarchaeon strains and could have applications in this field (O'Connor and Shand, 2002; Shand and Leyva, 2007). Currently, halocins are not considered for use as antibiotics in medicine, but some experiments showed that they could be used as compounds for the control of blood pressure and related pharmacological uses, including potential applications for the reduction of injury in organ transplantation (Meseguer et al., 1995; Alberola et al., 1998; Such et al., 1998; Lequerica et al., 2006; Shand and Leyva, 2007).

Other interesting compounds are siderophores, which are secreted by microorganisms and some plants. They are stronger iron-chelating agents that could be used in medicine for treatment of iron diseases or to increase antibiotic activity against bacteria, among others. They also have additional applications in agriculture. Dave et al. (2006) showed that five isolates from the species *Halococcus saccharolyticus*, *Halorubrum saccharovorum*, *Haloterrigena turkmenica*, *Halogeometricum* sp., and *Natrialba* sp. produced siderophores, which were identified as being of the carboxylate type.

In the search for new energy power sources, an ecologically friendly alternative is that of microbial fuel cells (MFCs), devices that convert chemical energy stored in organic substances or other reduced compounds into electrical energy by using microorganisms as biocatalysts. Considerable work has been done with MFCs using multiple combinations of microorganisms (Rabaey and Verstraete, 2005; Bullen et al., 2006; Davis and Higson, 2007).

Even halophilic bacteria have been used as biocatalysts in MFCs (Miller and Oremland, 2008). The use of haloarchaea as biocatalysts was proposed recently and it could have promising future applications for obtaining energy from saline wastewaters (Abrevaya et al., 2011b).

5.3.8. Applications in Astrobiology-Related Research

The particular features of halophilic archaea and the environment they inhabit makes these organisms interesting in such areas of research as astrobiology and exobiology. This is because haloarchaea in particular are able to tolerate many types of environmental stresses, not only high salt concentrations, but also high doses of UV or ionizing radiations, low levels of oxygen, and extremes of temperature and pH. These particular characteristics lead to proposing the use of these organisms as models for study in these areas because of their ability to survive extreme physicochemical conditions (DasSarma and DasSarma, 2005; DasSarma, 2006).

Moreover, the isolation of various species of haloarchaea found in inclusions in evaporitic rocks (salt rocks), particularly in old halites from different geological periods, has highlighted the apparent longevity of these organisms in brines. Among the most recent findings are those dating from the Permian and Triassic (Grant et al., 1998; McGenity et al., 2000; Radax et al., 2001; Vreeland et al., 2002; Mormile et al., 2003; Fendrihan et al., 2006). Because this type of evaporite has been detected in meteorites, including meteorites from Mars (SNC meteorites) (Gooding, 1992; Treiman et al., 2000; Rieder et al., 2004), these organisms have been proposed as potential inhabitants of Mars or other planetary bodies with saline environments, such as Jupiter's moon Europa (Rothschild, 1990; Grant et al., 1998; McCord et al., 1998; Landis, 2001; Mormile et al., 2003; Grant, 2004; Stan-Lotter et al., 2004; Fendrihan et al., 2006; Schubert et al., 2009).

Additionally, it has been suggested that Mars has an evaporitic environment and that the brine pockets formed as a consequence of the evaporation of water can harbor extant or extinct Martian biota (Mancinelli, 2005). This is supported by the recent detection of evaporite rocks of halite and sulfate carried out by NASA's rovers on the surface of Mars (Rieder et al., 2004; Tosca and McLennan, 2006) and by experiments showing the ability of these microorganisms to survive in simulated conditions similar to those of Mars (Fendrihan et al., 2009).

In another series of experiments the haloarchaea *Natrialba magadii* and *Haloferax volcanii* were exposed to simulated interplanetary conditions, in particular vacuum UV radiation at low Earth orbit, surviving at several doses (Abrevaya et al., 2011a). In experiments performed directly in Earth's orbit, the ability of the strain *Haloarcula* G to survive in space for several weeks, much longer than that for other vegetative cells, was shown (Mancinelli et al., 1998). This suggests this group of organisms as possible candidates for the interplanetary transfer of life by natural processes, although which are the central mechanisms that allow haloarchaea remain dormant for extended periods of time is unknown (Stan-Lotter et al., 2004).

Moreover, because halophilic archaea in general are exposed to high-UV doses in their natural environment, they are generally regarded as UV tolerant (DasSarma, 2006). It is believed that these microorganisms are highly resistant to the effects of UV damage, mainly because they have extremely efficient photoreactivation DNA repair mechanisms (Hescox and Carlberg, 1972; McCready and Marcello, 2003). If we compare the UV tolerance of

the haloarchaeon *Halobacterium* sp. NRC-1 to the UV tolerance of other organisms, it is possible to see that this strain is significantly more UV tolerant than that of several microorganisms even in the absence of photoreactivating mechanisms, due to the fact that haloarchaea, in general, also have DNA repair mechanisms (as excision repair) that are non-light dependent (McCready, 1996). These particular UV-resistant profiles were seen in experiments in which strains of *Natrialba magadii* were exposed to very high UV-C doses (a wavelength that does not reach the Earth's surface), simulating a possible exoplanet environment (Abrevaya et al., 2009).

Many microorganisms have been isolated from evaporitic deposits that date from 250 to 419 million years old (Denner et al. 1994; Stan-Lotter et al., 2001; Gruber et al., 2004). If these organisms can survive trapped in these salt crystals at times equivalent to these geological time scales, as suggested by several studies (Grant et al., 1998; McGenity et al., 2000; Stan-Lotter et al., 2004) and due to the fact that Mars and Earth may have had a similar geologic past (Nisbet and Sleep, 2001; Schidlowski, 2001), it is interesting to consider the possibility of a specific search for halophiles on Mars (Stan-Lotter et al., 2004; Abrevaya et al., 2010) or they may perhaps be considered as inhabitants of other planets, as their high UV survival profile suggests (Abrevaya et al., 2009).

5.4. CONCLUDING REMARKS

Many of the physiological, metabolic, and genetic features of haloarchaea are little known or still unknown. At the same time every year, new species are discovered in a diversity of environments, opening up a real field of research in the study of these microorganisms.

Due to there is still a lack of knowledge regarding some of these issues, and because the incorporation of some biological processes on an industrial scale are relatively new, applications of haloarchaea on a large scale are still very scarce, having overcome some obstacles specific to the area, such as the production of biomass, problems associated with purification procedures and costs. Many of their biotechnological applications have not yet been studied widely, although it is clear that haloarchaea and its by-products have a tremendous potential in different areas, as evidenced in this chapter. We can find applications related to their involvement in industrial processes as a source of new and more resistant enzymes, in the production of compounds related to the manufacture of various products (e.g., detergents, textiles, plastics), and in the production of fermented foods. Other uses are related to environmental applications in bioremediation, degradation of xenobiotics or hydrocarbons, or as part of a process for obtaining alternative power sources. There are also uses in electronics and in fields such as medicine.

It is important to note that while these organisms require large amounts of salt for optimum growth, in some cases their enzymes or by-products are not exclusively dependent on salt, which broadens the range of possible applications. Moreover, they are interesting as objects of study in research themselves and are even taken as models in growing fields of scientific research such as astrobiology.

In the next few years it will surely be possible to find more applications for haloarchaea and to improve some of the processes related to their applications. The sequencing of new genomes and the availability of new data from their proteomics and metabolomics will be very useful and will generate great advances in this field.

Acknowledgment

I would like to thank Dr. Eduardo Cortón and Pablo Nuñez Pölcher for the kind revision of the manuscript and helpful suggestions.

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BIOTECHNOLOGICAL APPLICATIONS OF COLD-ADAPTED BACTERIA

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6.1. INTRODUCTION

The biodiversity of severely cold habitats is wider than first predicted, as they have been colonized successfully by numerous organisms, including representatives from bacteria, archaea, and unicellular eukaryotes in addition to viruses. Cold environments represent the majority of the biosphere on Earth. The major fraction of low-temperature areas is represented by the deep sea (nearly 75% of Earth is covered by oceans and 90% of the ocean volume is below 5°C), followed by permafrost (24% of land surface), sea ice (13% of the Earth surface), and glaciers (10% of land surface). Living organisms have also been

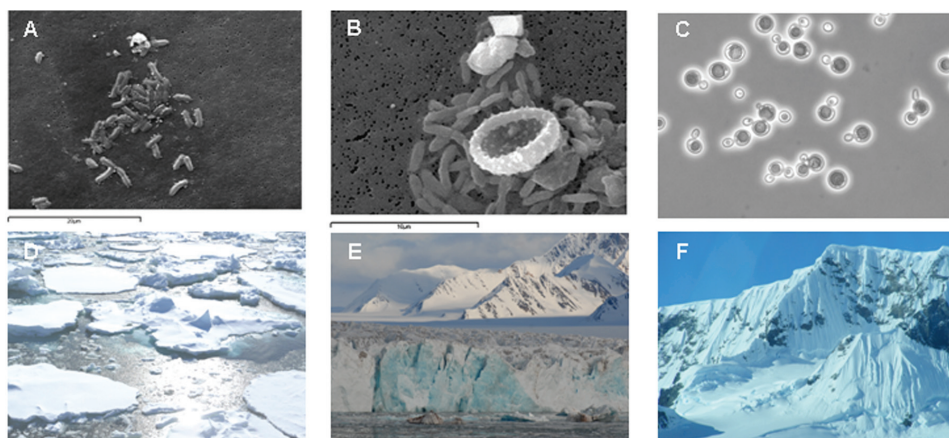


Figure 6.1. Psychrophiles in their natural habitats. Electron micrographs of *S. frigidimarina* from Antarctica (A), scanning electron micrographs of psychrophilic bacteria and unicellular eukaryotes from Arctic glaciers (B), optical micrograph of yeasts from ice samples (C), and pictures from their corresponding cold environments: Antarctic sea ice (D), Arctic glaciers (E), and high mountains in the Alps (F).

found in other permanently cold environments, such as deserts, lakes, caves, and the upper atmosphere (Fig. 6.1).

Microorganisms that live under cold conditions are generally known as *psychrophiles*, defined as organisms that have an optimal temperature for growth at about 15°C or lower, a maximal temperature for growth at about 20°C, and a minimal temperature for growth at 0°C or below. The lowest temperature limit for life seems to be around –20°C, which is the value reported for bacteria living in permafrost soil and in sea ice, although it has been reported that porous rocks in Antarctic dry valleys host microbial communities surviving at –60°C (Friedmann, 1982).

Cold-adapted microorganisms are sometimes classified as psychrophiles and psychrotrophs. The term *psychrotrophs* is used to designate psychrotolerant organisms that are not fully adapted to the cold and have an upper growth limit at temperatures higher than 20°C. An example of cell growth in these two types of bacteria is shown in Figure 6.2. Some authors believe that there is a continuum in their adaptation, depending on the environmental conditions; so the subdivision into two categories appears to be arbitrary.

Compared to other known prokaryotes, *psychrophiles* show many unique qualities and molecular mechanisms that allow them to adapt to cold environments. They have developed special adaptations at the level of all cellular constituents and molecular mechanisms to cope with the effects of low temperature and to avoid the destructive effect of intracellular ice formation.

Knowledge of the interaction of microorganisms in their environment is critical in accessing both a microorganism itself and processes that it uses to survive, both of which hold biotechnological promise. Biotechnology is recognized as one of the most promising

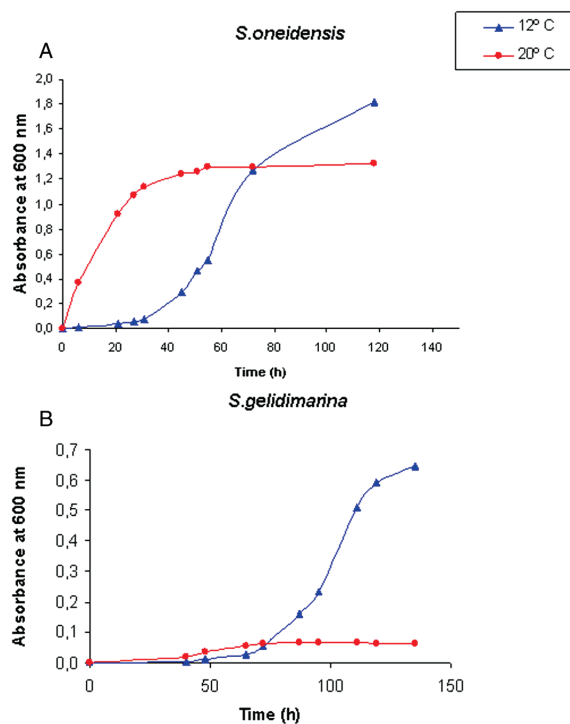


Figure 6.2. Bacterial growth of *Shewanella oneidensis* (A) and *Shewanella gelidimarina* (B) at either 12°C or 20°C. This experiment provides a comparison between a psychrotolerant (*S. oneidensis*) and psychrophilic (*S. gelidimarina*) species of *Shewanella*. In (A), cells that had been grown at 20°C were transferred to 12°C after being diluted with fresh medium. In (B), cells that had been grown at 12°C were transferred to 20°C. Cell growth was monitored by measuring the apparent absorbance at 600 nm. Each point represents the mean \pm S.E.M. of three experiments run in duplicate.

technologies for the twenty-first century because of its potential to ameliorate major global problems (disease, malnutrition, and environmental pollution), achieve industrial sustainability (optimizing the use of renewable resources, slowing global warming, and developing cleaner products and processes), and offer economic advantages. Since biotechnology is based on the discovery of exploitable biology, the recognition that only a small fraction of Earth's microbial biodiversity has been identified implies great potential for innovation (Bull et al., 2000).

The initial focus of “extreme biotechnology” aimed at thermophiles. However, it is generally recognized that although thermophiles have provided very important thermostable polymerase enzymes for polymerase chain reaction (PCR) (fundamental to most biotechnology), they have not fulfilled their promise in the broader sense of supplying new biotechnological tools. So there is also considerable interest in investigating the microbial forms that thrive in cold temperatures, especially those organisms that can supply biotechnological applications and have economic importance. Furthermore, they can provide important data

regarding the thermodynamic limits of life and the molecular mechanisms of adaptation to cold environments. Cold-adapted microorganisms and their enzymes, characterized by high catalytic activity and pronounced heat lability, function at temperatures as low as -20°C and have applications in a wide range of industrial applications: in the food, detergent, and cosmetic industries, from cold-water washing to molecular biology, as environmental biosensors, and in new drug discovery and the bioremediation of polluted cold soils and wastewaters.

6.2. MOLECULAR MECHANISMS OF ADAPTATION TO COLD ENVIRONMENTS

Cold-adapted organisms have successfully evolved features, genotypic and/or phenotypic, to surmount the negative effects of low temperatures and to enable growth in these extreme environments. The ability of psychrophiles to survive and proliferate at low temperatures implies that they have overcome key barriers inherent in permanently cold environments. These challenges include reduced enzyme activity, decreased membrane fluidity, altered transport of nutrients and waste products, decreased rates of transcription, translation and cell division, protein cold denaturation, inappropriate protein folding, and intracellular ice formation. Further, genes expressed at low but not at high temperatures are involved in conjugation and synthesis of flagella, among others. Psychrophiles are proving that the biochemical machinery of life can be adapted to conditions that from our anthropocentric perspective appear to be extreme. To thrive successfully in low-temperature environments, these microorganisms have evolved a complex range of structural and functional adaptations. By studying their molecular adaptations, we could try to identify the critical cellular components that limit life. Adaptations include the production of cold-active enzymes with high catalytic efficiency at low temperatures, the incorporation of unsaturated fatty acids in cell membranes to maintain membrane fluidity, the production of cold-shock proteins and cold-acclimation proteins to ensure improved protein synthesis at low temperatures, the synthesis of protective compounds to protect the cell from freezing (e.g., sugars, extracellular polysaccharides, antifreeze proteins), and the production of high amounts of antioxidant enzymes (catalase, superoxide dismutase, dioxygen-consuming lipid desaturases) for the detoxification of reactive-oxygen species (Gerday and Glansdorff, 2007).

As temperature affects essentially every aspect of an organism's physiology, from the basic structure of the macromolecules that are responsible for catalysis and information processing to the rates at which chemical reactions occur, different molecular mechanisms of biochemical adaptation to cold environments have been studied. For example, in their natural habitats, bacteria are frequently exposed to sudden changes in temperature and it has been shown that bacteria use different strategies to cope with temperature changes that are genetically determined and start with registration of the temperature followed by the induction of a subset of genes, allowing them to adapt to the stressful situation. These temperature changes are registered by three different thermosensors: DNA, RNA, and proteins (Schumann, 2009).

In addition, psychrophiles contain antifreeze proteins that have the ability to bind to ice crystals through a large complementary surface and thereby create thermal hysteresis and lower the temperature at which an organism can grow. Other molecules, such as

trehalose and exopolysaccharides (EPSs), also have an important role in cryoprotection, preventing protein denaturation and aggregation. Furthermore, high concentrations of EPSs modify the physicochemical environment of bacterial cells, take part in cell adhesion to surfaces and retention of water, favor the sequestration of nutrients, retain and protect extracellular enzymes against cold denaturation, and act as cryoprotectants (Mancuso Nichols et al., 2005).

6.3. EXOPOLYSACCHARIDES

Exopolysaccharides (EPSs) are high-molecular-mass carbohydrate polymers that make up a substantial component of the extracellular polymers surrounding microbial cells as a strategy for growth, adhering to solid surfaces, and to survive adverse conditions. Most EPSs produced by bacteria are heteropolysaccharides consisting of three or four different monosaccharides arranged in groups of 10 or less to form repeating units. These monosaccharides may be pentoses, hexoses, amino sugars, or uronic acids (Decho, 1990).

Typically, EPSs are either closely associated with the cell wall, forming a capsule, or loosely attached, forming slime. Although there are many possible functions for EPS, one general trend is that it increases cell survival by forming biofilms, retaining water (especially important in a frozen milieu), and serving as a cryoprotectant. Similar to the psychrophiles *Colwellia psychrerythraea* 34H and *Psychromonas ingrahamii* 37, *Psychrobacter arcticus* possesses genes for production of capsular-type EPSs. In our experiments EPSs have been investigated in *Psychrobacter frigidicola* to determine their structure. This bacterium, first isolated from Antarctica, forms a capsule in the presence of salt, suggesting that this could be an adaptation to growth in the permafrost environment. This adaptation had previously been described in *P. arcticus* isolated from Siberia, although taxonomic relatedness does not necessarily ensure similarity of EPS structure. A study by Mancuso Nichols et al. (2005) has shown that even among closely related strains, EPSs produced by Antarctic bacteria commonly found in cold environment were diverse. Ecological studies examining the role of EPSs provide evidence that these substances are abundant in arctic and antarctic environments (Mancuso Nichols et al., 2004, 2005). For example, *Pseudoalteromonas antarctica* NF3 also produces an exopolymeric compound of glycoprotein character that displays the ability to coat liposomes and provides protection against surfactants (Cocera et al., 2000, 2001).

In recent years there has been a growing interest in the isolation and identification of new microbial polysaccharides that may have novel applications as viscosifiers, gelling agents, emulsifiers, stabilizers, and texture enhancers. In the course of the discovery of novel polysaccharides of biotechnological interest, it has become widely accepted that psychrophiles will provide a valuable resource not only for exploitation in biotechnological processes, but also as models for investigating how biomolecules are stabilized when subjected to extreme conditions.

Indeed, EPSs produced by microorganisms from extreme habitats show biotechnological promise ranging from pharmaceutical industries, for their immunomodulatory and antiviral effects, bone regeneration and cicatrizing capacity, to food-processing industries for their peculiar gelling and thickening properties. Moreover, some EPSs are employed as biosurfactants and in detoxification mechanisms of petrochemical oil-polluted areas. Some

examples of EPSs produced by microorganisms isolated from cold environments and their applications are listed in Table 6.1.

6.4. LIPIDS

Low temperatures have an adverse effect on the physical properties of membranes, leading to a reduction in membrane fluidity. The lipid composition, which governs the physical properties of membranes, varies with the thermal habitat of microorganisms. So psychrophilic microorganisms modify the lipid composition of their membranes to maintain proper fluidity of the lipid bilayer. This involves the introduction of steric constraints, by reducing the packing order of acyl chains in the membrane. Such steric constraints destabilize the membrane and reduce lipid viscosity. This is achieved, for example, by synthesizing lipids with *cis*-unsaturated double bonds that induce a kink in the acyl chain, or by incorporation of branched lipids or of short fatty acyl chains that reduce the contacts between adjacent chains (Feller, 2007). This altered composition, which generally does not occur in other prokaryotes, is thought to have a key role in membrane fluidity by introducing steric constraints that change the packing order or reduce the number of interactions in the membrane (D'Amico et al., 2006).

Studies of psychrophilic bacteria from the antarctic genera *Shewanella* and *Colwellia* have shown that a high proportion of cold-adapted bacteria from sea ice possess the ability to produce polyunsaturated fatty acids, such as eicosapentaenoic acid or docosahexaenoic acid. These long-chain polyunsaturated fatty acids are used with pharmaceutical purposes, as these molecules have demonstrated numerous properties to fight against several diseases, such as atherosclerosis, diabetes, and high blood pressure.

6.5. PROTEINS

The completion of psychrophiles' genomes has facilitated the monitoring of global molecular changes at the proteomic level and the identification of protein biomarkers for cells undergoing a cold response. We have proved by proteomic techniques that main adaptations to cold at the protein level are the production of cold-shock proteins and cold-acclimation proteins to ensure improved protein synthesis at low temperatures and the production of cold-active enzymes (Fig. 6.3).

6.5.1. Stable Proteins at Cold Temperatures

Cold temperature affects multiple levels of cellular physiology, such as (1) a decrease in cytoplasmic membrane fluidity affecting membrane-associated functions, including active transport and protein secretion; (2) slow or inefficient folding of some proteins; (3) stabilization of secondary structures of RNA and DNA, leading to reduced efficiency of mRNA transcription and translation; and (4) adaptation of ribosomes to the low temperature to function properly (Schumann, 2009).

The exposure of mesophilic organisms to sudden temperature changes induces the transient overexpression of several proteins—known, respectively, as heat-shock proteins (Hsps) and cold-shock proteins (Csps)—that are involved in various cellular processes, such

TABLE 6.1. Biotechnological Applications of EPSs Produced by Microorganisms Isolated from Cold Environments

Microorganism	EPSs	Source Environment	Suggested Ecological Role and Application	References
<i>Pseudodalteromonas antarctica</i> NF ₃	Glycoprotein	Arctic marine environment	Coating liposomes, protection against surfactants and anticoagulant	Cocera et al., 2001
<i>Pseudodalteromonas</i> sp. (strain CAM025)	Sulfated heteropolysaccharide, high levels of uronic acids with acetyl groups	Sea ice from Antarctica	Cryoprotection	Mancuso Nichols et al., 2004
<i>Pseudodalteromonas</i> sp. (strain CAM036)	Sulfated heteropolysaccharide, high levels of uronic acids with acetyl groups	Particles from the Southern Ocean	Trace metal binding	Mancuso Nichols et al., 2004
<i>Pseudodalteromonas</i> sp. (strain SM9913)	Linear arrangement of α -(1 \rightarrow 6) linkage of glucose with a high degree of acetylation	Deep-sea sediment in the Yellow Sea, China	Flocculation and biosorption capacity	Qin et al., 2007; Li et al., 2008
<i>Psychromonas ingrahamii</i> 37	Not reported	Arctic sea ice, Alaska	Sequestering water/cryoprotection	Riley et al. 2008
<i>Psychrobacter arcticus</i>	Peptidoglycan	Siberian permafrost	Cell wall recycling	Das et al. 2011
<i>Colwellia psychrerythraea</i> (strain 34H)	Not reported	Arctic marine sediments	Cryoprotection	Marx et al., 2009
<i>Psychrobacter frigidicola</i>	Peptidoglycan	Sea ice from Antarctica	Cryoprotection	This study

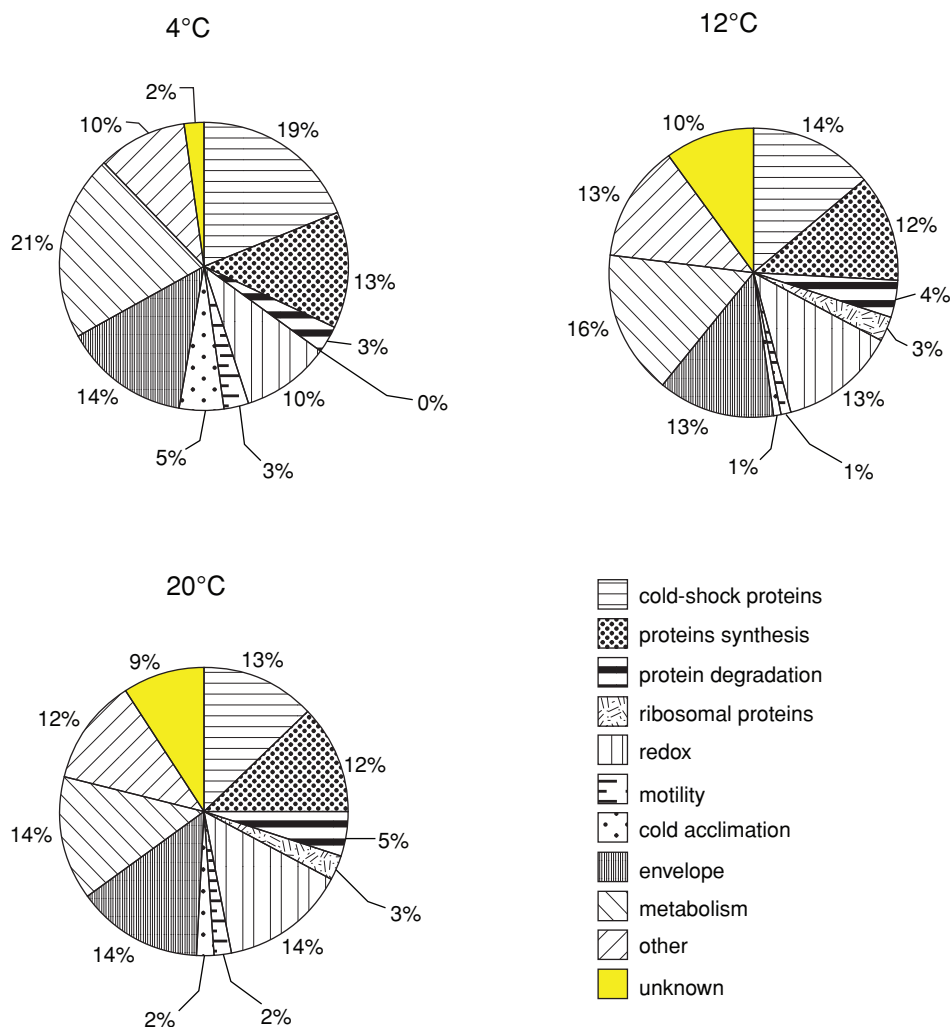


Figure 6.3. Protein distribution. Differentially expressed proteins identified by proteomic techniques in the psychrophilic bacteria *S. gelidimarina* growing at different temperatures: 4°C, 12°C, and 20°C. The main adaptations to cold at the protein level are the increase in the production of cold-shock proteins and cold-acclimation proteins and the production of cold-active enzymes.

as transcription, translation, protein folding, and the regulation of membrane fluidity. In particular, increased levels of nucleic acid-binding proteins (e.g., CspA-related proteins) and regulation through chaperones, such as HtpG, GroEL, and DnaK, have frequently been reported (García-Descalzo et al., 2011). However, distinctions do exist between the mesophilic and psychrophilic cold-shock response, including the lack of repression of housekeeping protein synthesis and the presence of cold-acclimation proteins (Caps) in psychrophiles. Many of the Csps observed in mesophiles act as Caps in psychrophiles, being expressed constitutively rather than transiently at low temperatures. Further, this differential regulation of expression indicates that a temperature sensory system and thermosensors at

the cell membrane level that sense changes in fluidity exist in psychrophiles (D'Amico et al., 2006).

6.5.2. Cold-Adapted Enzymes

Insight into cold adaptation has occurred through the studies performed on analysis of structures, biochemical activity, and stability of numerous enzymes from psychrophiles, also known as psychrozymes. A range of structural features correlates with enzyme cold adaptation. However, no structural feature is present in all cold-adapted enzymes, and no structural features always correlate with cold adaptation (Siddiqui and Cavicchioli, 2006).

Most biological reactions display a drop in activity on reducing the temperature from 37°C to 0°C. In contrast, psychrophilic microorganisms have been found to maintain relatively high metabolic fluxes at low temperatures, indicating that adaptation of the enzymatic repertoire has taken place to allow for appropriate reaction rates. Reported mechanisms of cold adaptation include an increased enzyme production (Crawford and Powers, 1992), and while an expression of specific isotypes adapted to different temperatures has also been reported (Lin and Somero, 1995), this suffers the disadvantage of requiring the presence of multiple gene copies and is most apt for organisms requiring seasonal adaptation. Finally, the synthesis of enzymes specifically adapted to operate at permanently low temperatures is another putative cold-adaptation mechanism, and this in fact appears to be the main physiological adaptation used by psychrophiles at the enzyme level.

Recently developed proteomic techniques for determining gene function along with differential expression detection and analysis have been applied as high-throughput technologies for function discovery and for reconstructing functional networks in psychrophiles. Their ability to proliferate in the cold is predicated on a capacity to synthesize cold-adapted enzymes, which have high specific activities and continue to function at near-freezing ambient temperatures, often up to an order of magnitude higher than those observed for their mesophilic counterparts. The commonly accepted hypothesis for this cold adaptation is the activity–stability–flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structure to compensate for the “freezing effect” of cold habitats. This increased flexibility might concern the entire protein or might be restricted to parts of the structure, especially those implicated in catalysis, compared to thermostable homologs (D'Amico et al., 2006).

The intimate linkage between conformational flexibility and protein function carries an important implication for the evolution of protein stability: To function quickly and with accuracy, proteins cannot become too rigid, at least in those regions of the molecule that are involved in recognizing ligands and that undergo changes in conformation during the catalytic cycle. Selection thus prevents proteins from acquiring the highest degree of structural stability that would be possible (Hochachka and Somero, 2002). For example, some of the main barriers to protein synthesis at low temperatures include reduced activity of transcriptional and translational enzymes, reduced protein folding, and a stabilization of DNA and RNA secondary structures. In psychrophiles, enzymes involved in these processes have adapted to be optimally active at low temperatures. Until now, more than 100 enzymes have been purified from prokaryotic organisms inhabiting permanently low-temperature environments: for example, cellulases, amylases, xylanases, DNA-ligases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases, alkaline phosphatases, lactamases, and phytases. Most of them can be consulted at <http://www.ulg.ac.be/biochlab> (Collins et al., 2008).

6.6. BIOTECHNOLOGICAL APPLICATIONS OF COLD-ADAPTED ENZYMES

Enzymes from extremophiles (or extremozymes) are especially useful since many industrial processes include procedures that require harsh conditions. The food, detergent, pharmaceutical, and paper industries, among others, are taking advantage of these enzymes to improve their processes. This is a relatively new research and development field where very much is to be done and very much is to be gained. Cold-active enzymes are beneficial not only for their enhanced selectivity and stereospecificity, but because they can also catalyze numerous reactions at low and moderate temperatures ($<40^{\circ}\text{C}$) more efficiently and with fewer undesired chemical reactions that may occur at high temperatures, thereby decreasing the overall energy expenditures and processing costs associated with heating steps (Cavicchioli et al., 2002).

6.6.1. Detergents

A variety of enzymes, including proteases, amylases, lipases, and cellulases, are used as part of detergents and personal care products. These enzymes present the benefits from developing detergents that hydrolyze soils and stains efficiently at low temperatures, reducing energy consumption and resulting in decreased associated costs and environmental impacts.

6.6.2. Food Industry

Cold-adapted enzymes are used for the processing of foods, due to their high catalytic activity at low temperatures, which minimizes spoilage and alterations in taste and nutritional values. For example, β -galactosidase is employed extensively in the manufacture of dairy products, including lactose-reduced milk. Some strains of cold-adapted bacteria such as *Rahnella* and *Buttiauxella* are able to break down lactose at low temperatures.

Cold-adapted lipases and proteases can lower production costs by accelerating the maturation of cheeses that require specific low-temperature and low-moisture conditions. Cold-adapted proteases can be used for tenderization and taste improvement of refrigerated meat products, and removal of undesirable tissues from seafood as well as in the brewing industry and animal feed production. Further, polygalacturonases, pectate lyases, and various hemicellulases, can be employed for the low-temperature degradation of pectin compounds in the fruit- and vegetable-processing industries, and enzymes such as xylanases, proteases, amylases, lipases, and glucose oxidases can modify the hemicellulose, gluten, starch, and free sulfhydryl groups, respectively, utilized in baking (Huston, 2008).

6.6.3. Pharmaceutical Industry

Cold-adapted organisms can also be exploited for the pharmaceutical industry. For example, bacteria from the genus *Pseudoalteromonas* are good sources of molecules with antibiotic activity (Feller et al., 1994). Several enzymes, such as hydrolases and lipases obtained from cold-adapted bacteria and yeasts, are being used in organic synthesis of complex drugs. Further, cold-active lipases have attracted attention because of their use in the synthesis of chiral intermediates at low temperature.

6.6.4. Biofuels

As human activity contributes to accelerate global warming, there is increasing interest in developing alternative sources of energy. Nowadays, biodiesel is considered a promising replacement to petroleum-derived diesel, and several studies have been developed to obtain biodiesel from antarctic microalgae to overcome the limitations that current biodiesel formulations present at low temperatures. Other biofuels, such as ethanol, also represent a renewable energy source. Cold-active esterase and β -glucosidase from psychrophilic bacteria are of considerable interest to enable cost-effective lignocellulose biomass conversion, thus facilitating the development of an economically viable ethanol production from agricultural waste, forestry waste, energy crops, and municipal solid waste.

6.6.5. Molecular Biology

The first cold-adapted enzyme from antarctic microorganisms to have been fully characterized was an alkaline phosphatase that offers a unique advantage, contrary to its mesophilic counterparts of being rapidly inactivated by mild heat treatment prior to the use of the kinase, thereby allowing higher yields. The coupling of oligonucleotides using cold-adapted DNA ligases also seems promising, as the ligation yield is much higher at low temperatures. Cold-adapted DNA ligases could offer a significant advantage over mesophilic enzymes poorly active at low temperatures (Georlette et al., 2004). In addition, psychrophilic hosts are being used as low-temperature expression systems which reduce the formation of inclusion bodies and increase the expression of correctly folded proteins under soluble form, due to the weakening of hydrophobic interactions at low temperatures.

6.7. BIODEGRADATION AND BIOREMEDIATION IN COLD ENVIRONMENTS

Of the many challenges faced for effective bioremediation strategies, microorganisms offer the potential to effectively and inexpensively clean up contaminated environments. Bioremediation attempts to accelerate the biodegradation rates through the optimization of limiting environmental conditions, such as temperature, nutrients, and bioavailability of contaminants. Pollution in cold climates is an area of particular importance, since contaminated areas are often remote, and thus the degradation capacity of indigenous microorganisms is required. Some biodegrading microorganisms from cold environments and their substrates for growth are listed in Table 6.2.

6.7.1. Biodegradation of Petroleum

The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of cold areas. Investigations of hydrocarbon spills in cold regions indicate that hydrocarbon degraders, typically bacteria, are widely distributed in polar soils and their numbers are usually enhanced following hydrocarbon spillage. These hydrocarbon-degrading bacteria present in contaminated polar soils typically belong to the genera *Rhodococcus*, *Sphingomonas*, and *Pseudomonas*.

TABLE 6.2. Biodegrading Microorganisms from Cold Environments and their Substrates for Growth

Substrate	Microorganism	References
<i>Bacteria</i>		
Hydrocarbon substrates: alkane and aromatic	<i>Acinetobacter</i> <i>Arthrobacter</i> <i>Pseudomonas</i> <i>Rhodococcus</i> <i>Sphingomonas</i>	Aislabie et al., 2006
Radionuclides	<i>Shewanella putrefaciens</i> <i>S. oneidensis</i> <i>S. algae</i>	Hau and Gralnick, 2007
Halogenated organics	<i>Shewanella putrefaciens</i> 200 <i>S. oneidensis</i> <i>S. algae</i> BrY	Hau and Gralnick, 2007
Nitramines	<i>S. sediminis</i>	Hau and Gralnick, 2007
Inorganic sulfur compounds	<i>Acidithiobacillus ferrivorans</i> SS3	Liljeqvist et al., 2011
<i>Yeasts</i>		
Phenol	<i>Rhodotorula psychrophenolica</i> <i>Trichosporon dulcitum</i> <i>Leucosporidium watsonii</i>	Margesin, 2007
Phenolic compounds	<i>Cryptococcus</i> <i>Rhodospiridium</i> <i>Rhodotorula</i> <i>Mastigobasidium</i> <i>Sporobolomyces</i>	Margesin, 2007
<i>Algae</i>		
Wastewater treatment	<i>Chlamydomonas</i> <i>Chlorella</i>	Grönlund et al., 2010 Cid et al., 2010

Several soils contaminated with petrol and corresponding pristine soils demonstrating the presence of significant microbial heterotrophic and oil-degrading cold-adapted populations have been compared in all the soils (Margesin et al., 2003). In these studies, the numbers of culturable cold-adapted hydrocarbon degraders were greater by up to four orders of magnitude than those of the corresponding mesophilic populations. Further, other studies demonstrated that hydrocarbon contamination results in a shift of the composition of soil microbial communities with an enrichment of gram-negative bacteria, especially members of γ -proteobacteria. Bioremediation strategies can involve biostimulation of the indigenous soil population and/or bioaugmentation (inoculation with efficient degraders).

6.7.2. Biodegradation of Phenolic Compounds

Cold-adapted degraders are also useful for wastewater treatment. Phenol and phenolic compounds are widely distributed as environmental pollutants, as they are common constituents of many industrial wastewaters, such as those produced from crude oil refineries and coal

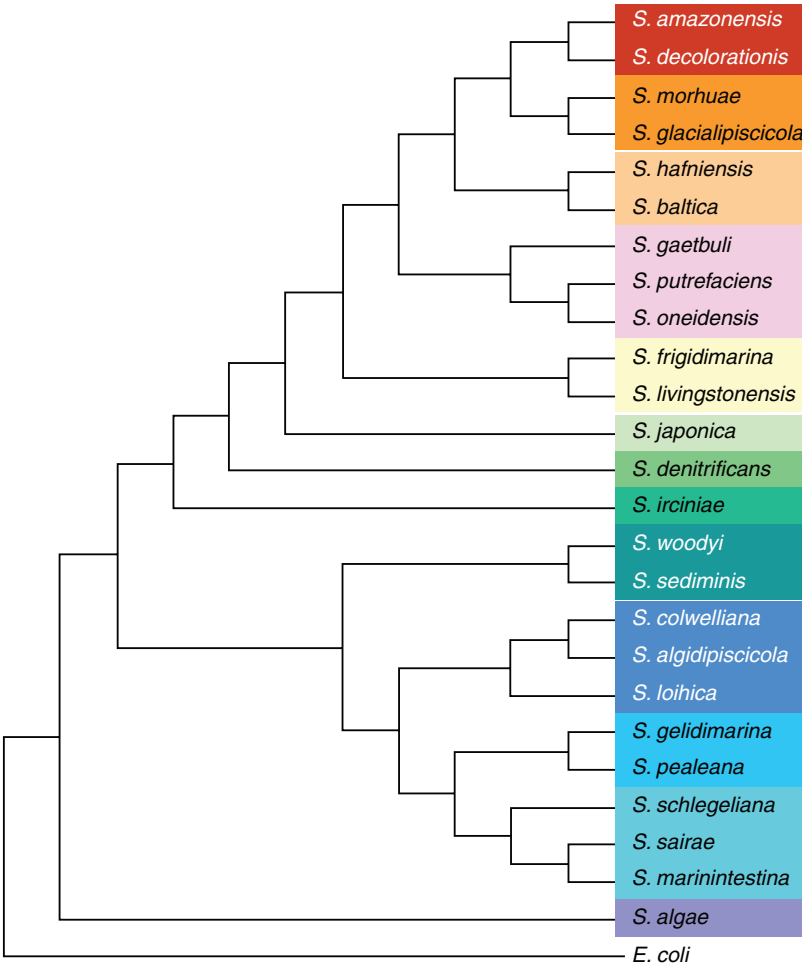


Figure 6.4. Phylogenetic tree of *Shewanella* 16 rRNA gene sequence from strains isolated from cold-temperature environments. Phylogenetic analysis was carried out by the neighbor-joining method using MEGA 5 program. (See insert for color representation of the figure.)

gasification plants. Psychrophilic methanogens play a very important role in the global carbon cycle. The application of psychrophilic methanogens in anaerobic biotreatment processes could greatly extend the application fields of anaerobic technology and reduce the operational cost of wastewater treatment.

6.7.3. Bioremediation of Radionuclides, Halogenated Organics, and Nitramines

Microorganisms represent one means by which changes in oxidation states of chemical compounds are catalyzed so that transport into rivers and groundwater can be blocked and

cleanup facilitated. As an example, the genus *Shewanella* has been widely studied because of its several biotechnological uses, such as bioremediation of chlorinated compounds, radionuclides, and other environmental pollutants. More than 50 strains of this genus have been isolated from various cold environments all around the world (www.bacterio.cict.fr). Phylogenetic relationships based on 16S rRNA gene sequences are represented in Figure 6.4 for several strains of *Shewanella*. Some shewanellae have demonstrated the ability to reduce insoluble forms of radionuclides, such as uranium, technetium, or cobalt, and have been suggested for use in remediation of contaminated environments and waste streams.

Further, associations of organisms and populations play key roles in the anaerobic biodegradation of chlorinated organic contaminants. For example, dehalogenation by the *Shewanellae* working in concert with a complex microbial consortium that converts toxic intermediates into benign or less toxic compounds in the environment has been reported. A unique feature of some *Shewanellae* strains that is not common to any other known bacteria is the ability to attenuate and/or degrade cyclic nitramines in contaminated marine sediments (Hau and Gralnick, 2007).

6.7.4. Treatment of Acid Mine Drainage

Effluents from mining operations treating sulfide ores often contain considerable concentrations of inorganic sulfur compounds, such as thiosulfate and tetrathionate. These drainages may cause environmental problems if released downstream due to oxidation to sulfuric acid. Toxicity experiments using mixed cultures from permanently cold environments demonstrated the potential of using biotechnological solutions to remove inorganic sulfur compounds at cold temperatures and thus, reduce the impact of mining on the environment (Liljeqvist et al., 2011).

6.8. CONCLUSIONS

Currently, only 1 to 2% of the microorganisms on Earth have been exploited commercially, and among these there are few examples of psychrophiles. Cold-adapted microorganisms and their molecular machinery offer many advantages in terms of their biotechnological potential and interesting alternatives to research sustainable resources. Many of these microorganisms live in remote areas where only a small fraction of the microbial biodiversity has been identified. Nowadays, the market demand for biotechnology and sustainable resources is increasing. Therefore, efforts have to be made to achieve a wider knowledge of psychrophiles' biodiversity and to develop genetically improved strains that would play an important role in sustainable industry and biotechnological applications.

Acknowledgments

We are indebted to Ms. Eva García-López for her technical assistance and to Drs. M. Martínez-Gomariz and C. Gil from Proteomic Unit of the Parque Científico de Madrid. This research was supported by grants CTM/2008-00304/ANT and CTM2010-12134-E/ANT from the Spanish Ministerio Ciencia e Innovación.

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- <http://www.bacterio.cict.fr>—list of prokaryotic names with standing in nomenclature.
- <http://www.ulg.ac.be/biochlab>—site to consult currently known enzymes isolated from psychrophilic organisms.

ECOLOGY AND BIOTECHNOLOGY OF EXTREMOPHILIC MICROORGANISMS, PARTICULARLY ANAEROBIC THERMOPHILES

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7.1. INTRODUCTION

A temperature of 37°C, a pH value around 7.0, salinity from 0.9 to 3%, and a pressure of 1 atm represent the ideal conditions for growing *Escherichia coli* and for most animals and human beings. These environmental parameters have always been referred to as normal or physiologic, but over the last century scientists have shown that a large number of organisms require more extreme conditions (Grant, 1988; Aguilar, 1996; Aguilar et al., 1998; Antranikian et al., 2005; Canganella and Wiegel, 2011). MacElroy coined the term *extremophile* in 1974 to describe these organisms, the majority of which are prokaryotic. Aside from intellectual curiosity, interest in studying extremophiles stems from their possible utility in industrial processes, their possible links to the origins of life on this planet, and possible clues as to how and where to look for extraterrestrial life (Stetter, 1996; Shock, 1997; Litchfield, 1998; Wiegel and Adams, 1998; Javaux, 2006; Lentzen and Schwarz, 2006; Villar and Edwards, 2006). A short summary of potential biotechnological applications for extremophiles is given in Table 7.1.

Extremophiles are best characterized by the minimum, maximum, and optimum parameters of the extreme condition for growth (i.e., the T_{\min} , T_{opt} , and T_{\max} for thermophiles). The physiology and taxonomy of extremophilic microorganisms are without doubt of great

TABLE 7.1. Biotechnological Application of Major Groups of Extremophiles

Extremophilic Organisms	Enzymes and Organic Compounds	Applications and Products
Thermophiles and Hyperthermophiles (T_{opt} 55–105°C)	Amylases	Glucose and fructose for sweeteners
	Xylanases	Paper bleaching
	Proteases	Amino acid production from keratins, food processing, baking, brewing, detergents
	DNA-polymerases	Genetic engineering
Psychrophiles and psychrotolerants ($T_{\text{opt}} < 20^{\circ}\text{C}$)	Neutral proteases	Cheese maturation, dairy production
	Proteases	Polymer-degrading additives in detergents
	Amylases	
	Lipases	
	Polyunsaturated fatty acids	Pharmaceuticals
Acidophiles ($\text{pH}_{\text{opt}} < 3$)	Dehydrogenases	Biosensors
	Sulfur oxidation	Desulfurization of coal, biomining of ores
Alkaliphiles ($\text{pH}_{\text{opt}} > 8.5$)	Cellulases	Polymer-degrading additives in detergents
	Proteases	
	Amylases	
	Lipases	Stabilization of volatile substances
	Cyclodextrines	
	Antibiotics	
Halophiles (growing with 3–20% salt)	Carotene	Food coloring
	Glycerol	Pharmaceuticals
	Compatible solutes	Pharmaceuticals
	Membranes	Surfactants for pharmaceuticals

interest, but these issues are not addressed here. In this chapter the ecology and biotechnology of various categories of extremophilic prokaryotes are discussed, particularly of anaerobic thermophilic microorganisms.

7.2. THERMOPHILES

These organisms are perhaps one of the most interesting varieties of extremophilic organisms because they can thrive at temperatures over 50°C. Among all thermophiles, there is a much higher number of anaerobes than aerobes. This is probably due to the fact that oxygen is much less soluble at higher temperatures and therefore is not available to organisms in metabolic processes. The main features of thermophilic microorganisms were described by several authors, focusing either on the thermophilic ecosystems, the systematics of thermophiles, or the physiological traits, including biotechnological applications (Brock, 1978; Wiegel, 1990, 1998; Kristjansson, 1992; Stetter, 1996; Amend and Shock, 2001; Wiegel

and Kevbrin, 2004; Burgess et al., 2007; Lebedinsky et al., 2007; Duncan et al., 2009; Hubert et al., 2010; Cowan and Fernandez-Lafuente, 2011; Littlechild, 2011).

Thermophilic microorganisms have been known for a long time, but it is always difficult to understand that some organisms do not only survive at high temperatures, but actually thrive in boiling water. Based on their optimal temperature, thermophiles can be subdivided into three main groups: moderate thermophiles, with an optimal temperature between 50 and 64°C and a maximum at 70°C; extreme thermophiles, with an optimal temperature between 65 and 80°C; and finally, hyperthermophiles, with an optimal temperature above 80°C and a maximum above 90°C (Stetter, 1996). The main interest in thermophiles during recent decades has been on issues dealing with basic and applied research. In addition, the discovery of many novel hyperthermophilic archaea (of which many can grow at 100°C and above and a few even up to 121°C) has attracted great interest among the scientific community. In this regard, a driving force for implementing basic and applied research on thermophiles was the realization that thermophilic microorganisms can serve as excellent sources for thermostable biocatalysts.

The finding of novel extremely thermophilic and hyperthermophilic bacteria in recent years, and the fact that most of them belonged to the archaea, definitely made this area of investigation more exciting. Therefore, it is not surprising that the majority of research work has been devoted to archaea in the last few years, despite the fact that enzymology and molecular biology are much more developed with thermophilic bacteria. One of the most astonishing reports was undoubtedly that on the description of *Nanoarchaeum equitans* (Huber et al., 2002, 2003), followed by its genome sequencing (Waters et al., 2003). The *Ignicoccus*–“*Nanoarchaeum*” system has been described as a symbiotic relationship: small cocci were attached to the larger cells of a strain of *Ignococcus* isolated from the Kolbeinsey Ridge, north of Iceland. These tiny cocci could be isolated from the larger cells and subsequently studied, but grew only when attached to their host. The achievement of the genome sequence analysis of *Nanoarchaeum* showed that it is missing most of the enzymes required for nonparasitic growth.

Due to the stress of living at such elevated temperatures, thermophiles have evolved a variety of mechanisms that allow them to survive at temperatures at which other organisms cannot thrive. These traits include unique membrane lipid composition, thermostable membrane proteins, and higher turnover rates for various protein enzymes. One of the most important attributes in the maintenance of homeostasis within the organism is that of the plasma membrane surrounding the organism. Archaeal thermophiles, and also acidophiles, have membranes containing unique ether lipids. These tetraether lipids span the entire membrane, forming a rigid monolayer that is impermeable to both ions and protons. Ether-type lipids such as these are more thermostable than the ester-type lipids found in nonthermophilic eubacteria and eukarya. Also, the lipid composition in the membranes of the thermophiles consists of more branched and saturated fatty acids than other organisms, to maintain the required correct lipid fluidity.

Aside from having to stabilize the plasma membrane at high temperatures, thermophiles must also stabilize their proteins, DNA, RNA, and ATP. The study of how they manage thermostability at the protein and membrane structural level has elucidated many traits of protein, membrane, and nucleic acid structure; however, there is not yet a full understanding of the principles of thermophily and thermostability of cell components. As a matter of fact, the process of heat stabilization for DNA, RNA, and ATP is not yet fully understood.

Regarding the heat stabilization of the proteins that are required for the maintenance of life, thermophiles have developed distinct ways of heat stabilizing the proteins: (1) the surface energy of the protein, along with the hydration of the nonpolar groups that are exposed, are minimized; (2) hydrophobic regions are packed into a very dense core of the protein by charge–charge interactions between amino acids; (3) salt bridges and other networks, which help to stabilize the structures at higher temperatures are increased; and (4) the synthesis of chaperonin proteins after a heat shock is markedly enhanced.

Organisms with optimal growth temperatures above 80°C, described as hyperthermophiles, contained in the deepest, least evolved branches of the universal phylogenetic tree, often use substrates which are thought to have been predominant in the primordial terrestrial makeup and produce substances that predominate in the present geochemistry. For these reasons they are particularly interesting from an evolutionary point of view and it is largely assumed that they could have been the first life forms on this planet (thus the name *archaea*) (Wiegel and Adams, 1998; Cherry, 2010).

Thermophilic anaerobes attract particular research attention because it is acknowledged that they have properties similar, in various aspects, to those of the early evolutionary life forms on Earth and thus possibly to extraterrestrial life. In this regard, a further and important interest in thermophiles concerns the issue of exobiology, and this is due primarily to the realization that thermophilic microorganisms and the microbial ecology of some terrestrial and marine extreme environments can be investigated as reference models for lunar and Martian environmental conditions.

7.2.1. Thermophilic Anaerobes and Clostridia

Microorganisms that grow optimally at elevated temperatures above 50°C and cannot use oxygen as a terminal electron acceptor during electron transport phosphorylation are described as thermophilic anaerobes. They are of interest from basic and applied scientific perspectives and are studied to understand how life can thrive in environments previously considered to be inhospitable to life. Such environments include volcanic solfataras and hot springs high in sulfur and toxic metals, as well as abyssal hydrothermal vents with extremely high pressure and temperature far above 100°C.

Besides natural thermal environments, thermophilic anaerobes are also found within anthropogenically heated environments, including coal refuse piles and compost heaps, which contain not only spore-forming species but also methanogenic archaea and nuclear power plant effluent channels. Contrary to any expectation, thermophilic anaerobes have also been isolated from mesobiotic and even psychrobiotic environments: *Thermosedimentibacter* species were isolated from ocean sediments at or below 12°C (Lee et al., 2005), and *Methanothermobacter thermoautotrophicus* as well as other thermophilic methanogens and chemolithoautotrophic acetogens were found in lake sediments and rivers, streams, and ponds (Wiegel et al., 1981). The presence of thermophilic anaerobes in environments where they were thought not to grow may be due to the fact that (1) the microorganisms are present but not growing in these environments, (2) they dispersed only transiently from other thermal environments, and (3) they are surviving and multiplying by taking advantage of temporary thermal piconiches.

The majority of thermophilic anaerobic archaea and eubacteria are chemoorganoheterotrophic, using organic compounds as energy and carbon sources. However, a variety of other metabolic strategies are seen within the set of thermophilic anaerobic

prokaryotes, including chemolithotrophy, photoorganotrophy, and photoautotrophy. With no doubt, an astonishing diversity of metabolisms is observed within thermophilic anaerobes. The Emden–Meyerhof and Entner–Doudoroff pathways are employed by glycolytic thermophilic anaerobes, but a variety of modifications have been discovered, predominantly within the archaea (Seliget et al., 1997). Major fermentation products formed by glycolytic thermophilic anaerobes include acetate, butyrate, lactate, ethanol, CO₂, and H₂ and to a lesser degree, propionate, propanol, and butanol; traces of various branched fatty acids from amino acid degradation are also detected since many glycolytic anaerobic thermophiles require yeast extract for growth and some even for metabolic activity.

Among anaerobic thermophiles, thermophilic clostridia have been largely investigated for many decades. The interest in these thermophiles first arose in the late 1920s and surfaced again in the late 1970s, due to the oil crisis. Since 1970, the number of thermophiles has increased tremendously. For example, in 1979, five clostridial thermophiles were known: *C. thermocellum* (since 1950 as pure culture), *C. thermosaccharolyticum*, *C. tartarivorum* (now regarded as a strain of *C. thermosaccharolyticum*), and *C. thermoaceticum*, all with a T_{\max} value around 65 to 69°C (moderate thermophiles), and *C. thermohydrosulfuricum* (with a T_{\max} of 75 to 78°C). More than 30 years later, the total number of taxonomically described clostridial thermophiles is not much higher, but their physiological and molecular traits have been largely elucidated.

Several of these species have been isolated only from hot springs, some only from mesobiotic environments or human-made environments, and others appear to be truly ubiquitous (e.g., *C. thermohydrosulfuricum*). Besides biotechnological applications in sewage treatment plants, only a very few organisms have been promising enough to be specifically included in patents, and most applications are either for alcohol production or as a source for thermostable hydrolytic enzymes (Canganella and Wiegel, 1993). No thermophilic clostridia are used in an established large industrial production process, probably because the renewed interest in their application is relatively recent, the concentration of products is too low, and/or the process of concentration and purification of the final product is often too energy demanding.

Last but not least, *Clostridium thermocellum* is the oldest validly published organism, and it is also the one that has so far drawn much attention for its potential in industrial applications.

7.2.2. Ecology of Thermophiles

The naturally hot environments on Earth range from terrestrial volcanic sites (including solfatara fields) with temperatures slightly above ambient, to submarine hydrothermal systems (sediments, submarine volcanoes, fumaroles, and vents) with temperatures exceeding 300°C, subterranean sites such as oil reserves, and solar-heated surface soils with temperatures up to 65°C.

Geothermal areas are mainly of three types: (1) acidic solfatara fields with abundant sulfur, acidic soils, acidic hot springs, and boiling mud pots; (2) freshwater hot springs and geysers with neutral-to-alkaline pH; or (3) deep-sea hydrothermal vents with neutral pH values and temperatures ranging between 20 and 350°C. Terrestrial geothermal areas can generally be divided into two classes according to the heat source. One type is the *high-temperature field* located within active volcanic zones and having a magma chamber



Figure 7.1. Power plant in Iceland. (See insert for color representation of the figure.)

at a depth of 2 to 5 km as a heat source. It is usually characterized by emissions of steam and volcanic gases on the surface with abundant hydrogen sulfide, which is then oxidized, first to sulfur and then to sulfuric acid. The other type of terrestrial geothermal area is the *low-temperature field*, located outside the volcanically active zone and heated by deep lava flows or by dead magma chambers. These sites are geologically more stable, and the individual hot springs are very constant in both temperature and water flow. Usually, the amount of hydrogen sulfide in the fluids is low and the same is true for carbon dioxide. Moreover, silica is precipitated with a resulting increase in pH, which is often steady around pH 9 to 10.

There are also human-made hot environments such as compost piles (usually around 60 to 70°C but as high as 100°C), slag heaps, industrial processes, and water heaters, as shown in Figure 7.1 (Oshima and Moriya, 2008). The deep sea is in general cold, but it is now known that there are areas of superheated water and widespread still-hot volcanic ocean crust beneath the flanks of the midocean ridge and other rock structures, as well as geothermally heated shallower ocean waters. Some representative natural hot environments are shown in Figure 7.2.

Many environments are temporarily hot, adaptation to which may be the reason that some thermophiles are very fast growing. Among the geothermally heated habitats are the alkaline, mainly carbonate-containing hot springs around neutral pH, and acidic areas including some mud holes. Most of the acidic high-temperature habitats contain elemental sulfur and metal sulfides, and most isolates from these areas metabolize sulfur by either anaerobic respiration or fermentation. Ocean depths are under extreme pressures from the weight of the water column, and thus most isolates from these areas are piezo-tolerant; some are truly piezophilic; others, such as *Pyrococcus* strain ES4 and *M. kandleri*, show extensions of T_{\max} under increased pressure (Pledger et al., 1994; Summit et al., 1998; Takai et al., 2008); and all are at least halotolerant (Adams, 1999), while those isolated from solfataras are generally acidophilic. While most species of described obligately aerobic thermophilic archaea are acidophilic, the anaerobic thermophilic bacteria are generally



Figure 7.2. Some representative hot environments: (A) fumarole at Campi Flegrei near Napoli, Italy; (B) terrestrial hot springs at Viterbo, Italy; (C) deep-sea hydrothermal vents at Okinawa Trough in Japan. (Courtesy of F. Canganella.) (See insert for color representation of the figure.)

unable to grow at acidic pH, but many anaerobic bacteria and some archaea grow at alkaline pH (Wiegel, 1998). The anaerobic alkalithermophilic bacteria thus form an interesting group to study.

7.2.3. Ecology of Extreme Thermophilic Archaea

Extreme environments have been described extensively with research focused primarily on physicochemical parameters such as salinity, pH, hydrostatic pressure, and temperature. Within the archaea only some of the neutrophilic strictly anaerobic methanogenic thermophiles have been isolated from all anaerobic habitats, at low and elevated temperatures. A few of the extreme thermophilic methanogens (e.g. *Methanobacterium* spp.) have been found in sewage treatment systems and lake sediments, but most have been isolated from hot springs (including deep-sea hydrothermal vents).

All members of the sulfur-utilizing archaea have been isolated only from geothermal areas. The aerobic genus *Sulfolobus* is also acidophilic, growing optimally at pH 2 to 3. This is in accordance with its metabolism (oxidizing sulfur to sulfuric acid) and its natural habitats (hot acidic springs and soils). The sulfur-metabolizing anaerobes, on the other hand, are moderately acidophilic or neutrophilic, most growing optimally at pH 5.5 and some as high as pH 7.0. Since they are either obligate or facultative sulfur-reducing microorganisms, they must grow as close as possible to the surface, where elemental sulfur is accumulated from the oxidation of hydrogen sulfide. Their environment thus remains mildly acidic, as the sulfur is further oxidized to sulfuric acid on the oxic side of the layer, and the acid partially diffuses to the anaerobic side. Most of the anaerobic extremely thermophilic archaea have an optimum pH of growth between 5 and 6.5.

7.2.4. Ecology of Extreme Thermophilic Bacteria

In terms of metabolism and habitats, the ecology of eubacteria is more complex than that of archaea due to the larger diversity existing among its presently known members. Aerobic spore-forming bacilli such as *B. caldolyticus* and *B. caldotenax* utilize a variety of carbon sources, such as glucose, succinate, pyruvate, and acetate when grown at 70 to 75°C, but only pyruvate if they are cultivated at 80°C. Both species were isolated from Yellowstone National Park from a site with a constant temperature of 86°C. Isolation of similar strains from other ecosystems has not yet been reported.

Extreme thermophilic bacteria can easily be isolated from hot springs but also from a number of other habitats that are heated only temporarily or from places that are permanently exposed to low temperatures. Neutral and alkaline hot springs are the main habitats of non-spore-forming extreme thermophilic aerobes such as *Thermothrix thiopara*, *Thermus thermophilus*, *Thermus filiformis*, *Thermomicrobium roseum*, *Hydrogenobacter thermophilus*, and *Calderobacterium hydrogenophilum*; however, some of these microorganisms have also been isolated from hot pipes or factories, such as species of *Thermus*. Most of these thermophiles are obligate aerobes except for some strains that can grow anaerobically with nitrate as an electron acceptor, forming either nitrite or nitrogen gas. As with extreme thermophilic archaea, aerobic bacteria growing optimally between 70 and 90°C are found primarily at neutral or alkaline pH; none have yet been isolated from acidic solfataras.

The order Thermotogales (Huber and Stetter, 1998) forms a special class among eubacteria and contains the most thermophilic bacteria known at present. They occur in shallow and deep marine hydrothermal systems as well as in continental solfataric springs with low salinity. *Thermotoga maritima* ($T_{\text{opt}} = 80^{\circ}\text{C}$) and *Thermotoga neapolitana* ($T_{\text{opt}} = 80^{\circ}\text{C}$) represent two extreme thermophilic species within the Thermotogales (Huber and Stetter, 1998). The former is the type species and was originally isolated from a shallow geothermally heated marine sediment at Vulcano, Italy. The latter was obtained from a submarine thermal vent at Lucrino near Naples, Italy, and from continental solfataras with low ionic strength at Lac Abbé, Djibouti, Africa. The genus *Thermosipho*, presently composed of seven described species, has as a reference species *Thermosipho africanus*, which has been described taxonomically and was also isolated from shallow hydrothermal springs and hot sandy marine sediments (Gulf of Tadjoura, Djibouti, Africa).

7.2.5. Biotechnology of Thermophiles

Thermophiles are certainly interesting in terms of biotechnologies, as many chemical industrial processes employing high temperatures have to be lowered in order to use bioprocesses from mesophiles, and this could be avoided using enzymes of thermophiles (Wiegel and Ljungdahl, 1986; Canganella and Wiegel, 1993; Woods, 1993; Huber and Stetter, 1998; Kube et al., 2006; Hong et al., 2009; Kumar et al., 2009; Zhong et al., 2009; Chang and Yao, 2011; Cowan and Fernandez-Lafuente, 2011). Research into thermophilic microorganisms has demonstrated that proteins from thermophiles are generally more stable than other proteins, and retain this property when cloned and expressed in mesophilic bacteria, although some mesophilic microorganisms can have enzymes being active at 100°C, and enzymes from thermophiles that are in purified form in vitro are not stable at the upper growth temperatures of the microorganism.

Further biotechnologies involving thermophiles investigated cover the fields of biomass and complex organic molecule degradation (Blumer-Schuetz et al., 2008; Vanfossen et al., 2008; Cavinato et al., 2010; Creamer et al., 2010; Kim and Lee, 2010; Suryawanshi et al., 2010; Sizova et al., 2011), metal leaching (Chen and Pan, 2010), production of compatible solutes (Empadinhas and da Costa, 2006), and water treatment technology (Liao et al., 2010). The potential application of thermophilic microorganisms for the production of biofuels was investigated in particular again in the last decades, mainly as research activities on the metabolism of pure or mixed cultures to produce biofuel, including methane and hydrogen, but also throughout extensive lab work with the aim of obtaining ethanol from biomass by means of thermophilic biological processes (Wiegel, 1980; Henstra et al., 2007; Koskinen et al., 2008; Shaw et al., 2008; Taylor et al., 2009; Kongjan et al., 2010; Roberts et al., 2010).

Research into thermophilic microorganisms has demonstrated that thermo-tolerant proteins are generally more stable than other proteins and retain this property when cloned and expressed in mesophilic bacteria (Connaris et al., 1998; Hayakawa et al., 2009). Enzymes active and stable at elevated temperatures were investigated for biotechnological applications, particularly for bioconversion, biorefining, and biofuel production (Donaghy et al., 2000; Vielle and Zeikus, 2001; Li et al., 2005; Razvi and Scholtz, 2006; Turner et al., 2007; Liang et al., 2010; Littlechild, 2011).

Much research on extreme thermostable enzymes has focused on their ability to degrade biopolymers such as cellulose and hemicelluloses, and such activities were largely supported by the development of novel techniques and devices to obtain high biomass yields (Figures 7.3 to 7.5). Together with chitin, these represent the most abundant



Figure 7.3. The Deep-Bath system developed and engineered at Jamstec in Japan for the continuous cultivation of extremophiles under elevated hydrostatic pressure. (Courtesy of F. Canganella.)



Figure 7.4. Titanium fermentor developed and engineered at Regensburg University in Germany for the continuous cultivation of sulfur-utilizing extremophiles at elevated temperature. (Courtesy of Harald Huber.)

biological polymers on Earth, but so far little investigation has been carried out on the degradation of chitin by extremely thermophilic microorganisms. The large majority of cellulolytic thermophiles described so far are anaerobic bacteria. However, it is not clear whether this is due to a restricted biodiversity or to nonoptimized isolation techniques.

The second important group of enzymes from extreme thermophiles includes amylases and pullulanases, which have been proposed for applications in the food industry, particularly for the production of starch-derived syrups. In addition, proteases, chitinases, and pectinases are potentially useful candidates. For the production of starch-derived syrups, α -amylases, glucoamylases, pullulanases, α -amylases, and glucose-isomerases are used. The processing is performed over the temperature range 55 to 105°C and at pH values from 4.5 to 8.0.

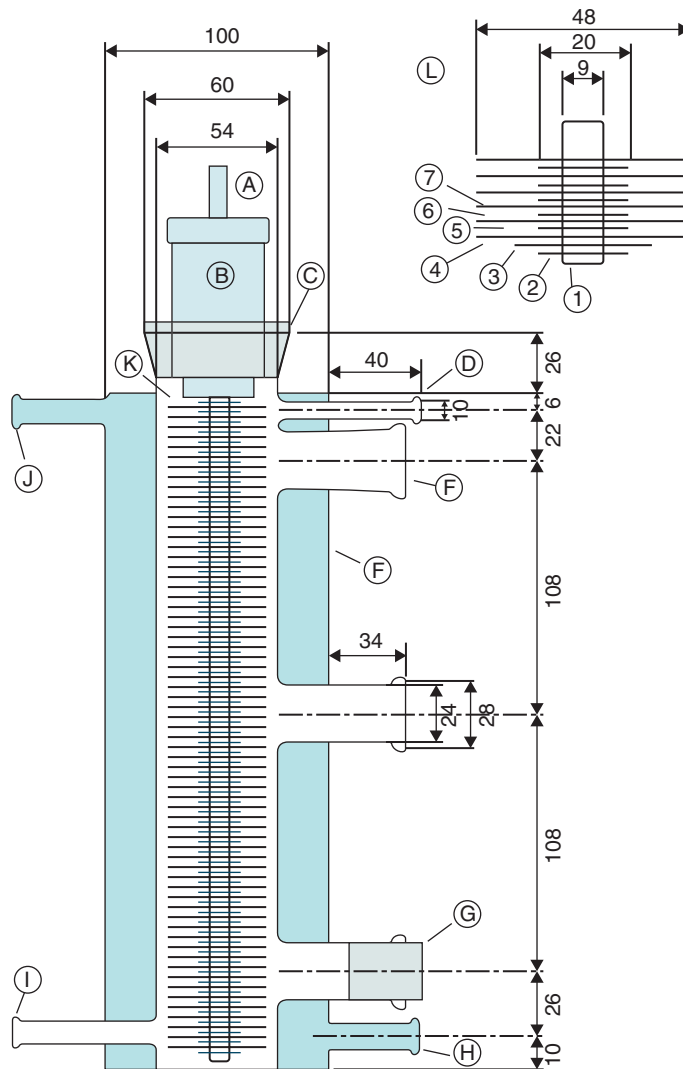


Figure 7.5. The rotary fermentor developed and engineered at the University of Georgia for the continuous cultivation of microorganisms to achieve high biomass yields. (Courtesy of J. Wiegell.)

Pectate lyases were described by Kozianowski et al., (1997) from *Thermoanaerobacter italicus*. These heat-stable enzymes represent the first pectate-lyases isolated from a thermophilic anaerobic microorganism. In the area of thermostable proteolytic enzymes, a protease and a caldolysin from two *Thermus* strains have been investigated for meat tenderization. In this case, analyses of temperature–activity relationships and sensory results showed that specific concentrations of both enzymes improved the tenderness significantly. Thermostable proteases, with selective catalytic activities against chicken feathers, have also been isolated by Friedrich and Antranikian (1996) from *Fervidobacterium pennavorans*, an extreme thermophile isolated from thermal springs in the Azores islands off Portugal.

The molecular structure of thermostable enzymes has been investigated extensively by point mutation analyses and by comparing their configuration with mesophilic homologous counterparts to determine the key features of their thermostability. A number of enzymes were analyzed in *T. maritima*, and it has become clear that the excess free energy of stabilization is equivalent to only a few weak bonds. However, so far no general rules for protein thermostability have been established. Comparison of thermostable enzymes with mesophilic counterparts showed that all enzymes contain a significantly increased number of charged amino acid residues and may therefore be stabilized by additional salt bridges. The most striking differences usually include the formation of additional hydrogen-bonding networks involving both side- and main-chain atoms. Folding and unfolding of thermostable proteins also represent important features that might give new insights into their unique catalytic properties.

All experimental evidence suggests that the differences in structures and functions between very stable and less thermostable enzymes are relatively small and are comparable with those differences found among enzymes of similar stability. The results seem to indicate that protein degradative reactions at high temperatures occur only slowly in conformationally intact proteins, rendering conformational stability to be the fundamental trait limiting the upper temperature for enzymatic activity. The interrelationships among conformational stability, enzymatic activity, and protein flexibility suggest that we cannot expect to find native enzymes that are stable far above the maximal growth temperature of the microorganism from which they originated. A significant enhancement of conformational stability might be achieved by genetic engineering, but each enzyme will probably require a specific molecular approach.

The production of ethanol by glycolytic and cellulolytic taxa has always been a major research subject. Cellulose and hemicellulose are the most abundant renewable natural plant fibers, and their degradation, coupled with the production of “biofuels” such as ethanol by thermophilic anaerobes, has been an intensely studied and timely research area for the last 40 years. Such developments occurred although research on fuel production leading to patents was done as early as the late 1920s, including a description and use of the oldest validly published anaerobic thermophile, *Clostridium thermocellum*. Recently, the focus was shifted to butanol and H₂ production, involving both thermophilic anaerobes and clostridia, particularly for the bioconversion of agrofood biomass (Talabardon et al., 2000; Verhaart et al., 2010; Carver et al., 2011; Wang et al., 2011; Xi-Yu and Chun-Zhao, 2011; Xu and Tschirner, 2011).

As with cellulose-degrading thermophilic anaerobes, xylanolytic thermophilic anaerobes generated further interest because the conversion of xylan—a component of plant hemicellulose and the second-most abundant renewable polysaccharide in biomass—to useful products might be coupled to increasing the efficiency of processing lignocellulose, and to the production of energy from renewable resources. Xylan is widely used as a carbon and energy source among thermophilic anaerobic Eubacteria, especially among members of the Firmicutes. Xylanolytic strains without cellulolytic activity are of interest in the pulp and paper industry, especially if they carry small (<30 kDa) xylanases. Lipolytic chemoorganotrophic thermophilic anaerobes include the anaerobic *Thermosyntropho lipolytica*, the first facultative thermophilic syntroph which, in coculture with effective H₂ utilizers, grows syntrophically on saturated and unsaturated fatty acids with 4 to 18 carbon atoms containing two of the most thermoactive and thermostable lipases (Salameh and Wiegel, 2010). Although chemoorganotrophic metabolisms appear to be common metabolic strategies for the

thermophilic anaerobes studied in the lab, the natural substrates for many of these microorganisms are largely unknown since very few *in vivo* experiments with isotopically labeled substrates have been carried out. On the other hand, culture-independent analyses indicate that chemolithoautotrophic prokaryotes are the primary producers in certain thermobiotic environments, such as hot springs (Bhaya et al., 2007).

Regarding gaseous biofuels and wastes, chemolithoautotrophic pathways are of interest, particularly the methanogenic reaction $4\text{H}_2 + \text{CO}_2 \rightarrow \text{CH}_4 + 2\text{H}_2\text{O}$, which is well characterized and used by thermophilic taxa within the Methanobacteriaceae, Methanothermaeae, Methanocaldococcaceae, and Methanococcaceae. Another interesting chemolithoautotrophic metabolism of anaerobic thermophiles makes use of CO, which occurs as a normal component of escaping volcanic gas of terrestrial and deep-sea hydrothermal origin. Several thermophilic anaerobes have indeed been isolated that grow lithotrophically on CO, performing the metabolic reaction $\text{CO} + \text{H}_2\text{O} \rightarrow \text{CO}_2 + \text{H}_2$.

Applied research activities on thermophilic and hyperthermophilic microorganisms, particularly anaerobes, have led to the development of novel methods, processes, and production systems. In Table 7.2 some representative patents concerning thermophilic anaerobic processes, microorganisms, and their products are reported.

7.3. ACIDOPHILES

The term *acidophiles* is generally used to describe organisms that can grow at pH values lower than 5 and that show pH optima between 2 and 4. The fact that microorganisms growing at pH values close to zero have been described (Schleper et al., 1995) will certainly expand further the group of extremely acidophilic organisms.

Most acidophiles have evolved extremely efficient mechanisms to keep the cytoplasm at or near neutral pH, and several processes are associated with pH homeostasis in acidophiles (Johnson, 1998; Dopson et al., 2004; Baker-Austin and Dopson, 2007; Booth, 2007):

1. Acidophiles reverse the reversed membrane potential to partially deflect the inward flow of protons. One potential mechanism of generating a reversed membrane potential is by potassium transport, a predominance of potassium-transporting ATPases is found in acidophile genomes.
2. Many acidophiles have evolved highly impermeable cell membranes to retard the influx of protons into the cell.
3. ΔpH is maintained through active proton export by transporters.
4. The sequencing of several acidophile genome sequences has indicated that there is a higher proportion of secondary transporters than in neutralophiles. Overall, they reduce the energy demands associated with pumping necessary solutes and nutrients into the cell.
5. The presence and availability of enzymes and/or chemicals capable of binding and sequestering protons might help to maintain pH homeostasis.
6. Comparative genome analysis suggests that a larger proportion of DNA and protein repair systems might be present in acidophiles compared with neutralophiles and that this could be associated with the cellular demands of life at low pH.
7. Organic acids that function as uncouplers in acidophiles might be degraded by heterotrophic acidophiles.

TABLE 7.2. Representative Patents Concerning Thermophilic Anaerobic Processes, Microorganisms, and Their Products

Publication Number	Applicant	Title	Topic
WO 2011/038393 A2	The Trustees of Dartmouth College	Cellulose and xylan fermentation by novel anaerobic thermophilic clostridia isolated from self-heated biocompost	A novel species of thermophilic clostridium is described and proposed for conversion of biomass to bioconversion products
WO 2011/076797 A1	Biogasol IPR APS	Thermophilic <i>Thermoanaerobacter italicus</i> subsp. <i>marato</i> having high alcohol productivity	Use of <i>Thermoanaerobacter italicus</i> subsp. <i>marato</i> and its mutants to produce fermentation products from lignocellulosic biomass
WO 2011/071829 A2	Mascoma Corp.	Heterologous expression of urease in anaerobic thermophilic hosts	Achievement of heterologous expression of urease in anaerobic thermophiles in order to use urea as a nitrogen source in bioprocessing systems
US 2011/0281362 A1	Mascoma Corp.	Electrotransformation of gram-positive anaerobic thermophilic bacteria	Development of methods for transforming gram-positive anaerobic thermophilic bacteria via electroporation with the result of increased transformation efficiencies
US 2010/0009420 A1	Oliff & Berridge, PLC	Genetic modification of homolactic thermophilic bacilli	Development of a method for modifying moderately thermophilic <i>Bacillus</i> species that are facultative anaerobic and homolactic by genetic engineering
CA 2010/01/29 A1	Renewable Energy Alternatives LLC	A system and method for a thermophilic anaerobic digester process	Proposal of a new approach that contemplates systems and methods to support environmentally friendly “green” thermophilic anaerobic digestion processes
US 2009/0029410 A1	Novozymes North America	Lipases from thermophilic anaerobes	Identification and application of polypeptides having lipase activity in anaerobic thermophilic bacteria
US 2009/0209025 A1	Woodard, Emhardt, Moriarty, McNett & Henry LLP	High solid thermophilic anaerobic digester	Development of a device for digesting sludge anaerobically and at elevated temperature
US 2008/0073266 A1	McQuaide Blasko	Continuous multistage thermophilic aerobic and aerobic–anaerobic sludge treatment process	Development of a continuous multistage treatment for sludge, comprising aerobic–anaerobic and thermophilic stages

WO 2006/039508 A2	Mixing and Mass Transfer Technologies LLC	Continuous multistage thermophilic aerobic and aerobic-anaerobic sludge digestion systems	Development of a continuous multistage digestion system for sludge, comprising aerobic-anaerobic and thermophilic stages
US 2005/0026293 A1	Merchant & Gould PC	Plasmids from an extremely thermophilic microorganism and derived expression vectors	Isolation of plasmids from extremely thermophilic anaerobic microorganisms as tools for creating novel shuttle vectors
WO 2004/035491 A1	Kemijski Institute	Procedure and device for thermophilic temperature range stabilization and mineralization of sludge from wastewater treatment plants	Development of a process for thermophilic two-stage anaerobic stabilization and mineralization of waste sludge, including thermal regeneration
WO 2003/016536 A2	Technical University of Denmark	Plasmids from an extremely thermophilic microorganism and derived expression vectors	Isolation of plasmids from extremely thermophilic anaerobic microorganisms and their use for genetic transformation systems
FR 2002/849019 A1	Ondeo Degremont	Procedure and tool in order to reduce the production of sludge from wastewater treatment plants	Development of a aerobic-anaerobic process for water treatment, including thermophilic enzymes
CA 2001/341168 C	Elsworth Biotechnology Ltd.	Process for ethanol production using thermophilic bacterium	Development of a process for the production of ethanol using <i>B. stearrowthermophilus</i> or thermophilic facultatively anaerobic bacteria
US 1997/5624841	Microbiological Research Authority of the Centre for Applied Microbiology & Research, UK	Process for the production of thermophilic microorganisms in high yield	Development of a process for the growth of anaerobic thermophilic microorganisms such as <i>Pyrobaculum islandicum</i> and <i>Pyrococcus furiosus</i> to high cell densities

(continued)

TABLE 7.2. (Continued)

Publication Number	Applicant	Title	Topic
US 1997/5630942	Purification Industries Int.	Two-phase anaerobic digestion process utilizing thermophilic fixed-growth bacteria	Improvement of an anaerobic wastewater treatment process, including thermophilic fixed-growth hydrolytic and acidogenic bacteria
WO 1996/04366	French Institute of Scientific Research for Development and Cooperation	Non-sulfato-reducing strict anaerobic thermophilic and hyperthermophilic bacteria culture method	Development of a culture method involving non-sulfato-reducing strict anaerobic thermophilic and hyperthermophilic bacteria for the production of enzymes
WO 1991/13996 A1	Rijks University, NL	Method for obtaining an anaerobic thermophilic bacterium, thus obtainable bacterium and its use for the fermentation of carbohydrates	Isolation and characterization of an anaerobic thermophilic bacterium capable of producing succinate
US 1988/4778760	Itachi Ltd. Japan	Thermostable α -amylase producing thermophilic anaerobic bacteria, thermostable α -amylase and process for producing the same	Isolation and application of a thermostable α -amylase produced by a <i>Clostridium</i> strain
US 1987/4651759	Philip Morris Inc.	Startup process for the thermophilic denitrification of tobacco	Development of a high-temperature process for the denitrification of tobacco involving thermophilic organisms
EU 1984/0139546	Institute Pasteur, France	Procedure for the production of alcohol through the fermentation of thermophilic bacteria, under elevated concentrations of organic solvents	Production of alcohol by microbial fermentation, involving thermophilic microorganisms

Studies of proteins adapted to low pH have revealed a few general mechanisms by which, for example, proteins can achieve acid stability. In most acid-stable proteins (such as pepsin and the soxF protein from *Sulfolobus acidocaldarius*), there is an overabundance of acidic residues which minimizes low-pH destabilization induced by a buildup of positive charge. Other mechanisms include minimization of solvent accessibility of acidic residues or binding of metal cofactors.

At high temperatures some microorganisms tend to use a higher proportion of purines in their codons, which are more resistant than pyrimidines to heat denaturation, but unfortunately, at low pH, purines are highly susceptible to acid hydrolysis. Some thermophilic acidophiles have adapted to growth at high temperatures by a general increase in the concentration of purine-containing codons as a heat-stabilizing adaptation while simultaneously reducing the concentration of purine-containing codons in long open reading frames that are more prone to acid-hydrolysis-associated mutations.

The genus *Acidianus*, which was first described by Segerer et al. (1986), is a member of the order Sulfolobales (Stetter, 1989) and comprises three formally recognized species of facultatively aerobic thermoacidophiles isolated from geothermal or hydrothermal systems (Plumb et al., 2007).

7.3.1. Acidophilic Ecosystems

Acidic habitats are abundant, and there are a number of natural processes that result in net acidity. Representatives among these may be several types of prokaryotic metabolism, including nitrification, accumulation of organic acids during fermentative or aerobic metabolism, and the oxidation of elemental sulfur, reduced sulfur compounds, and ferrous iron (especially in the form of pyrite). Some soils of volcanic origin, such as in solfataras and fumaroles, are generally acidic and rich in elemental sulfur or sulfidic ores, as are many hot springs and areas that surround them. However, the majority of extremely acidic habitats are at least partially anthropogenic, owing their existence to one particular human activity, the mining of metals and coal (Johnson, 1998; Bond et al., 2000). These habitats include coal refuse piles, abandoned mine shafts or pits, copper leaching dumps, and soils, rivers, or lakes contaminated by acidic runoff from these sites.

7.4. ALKALIPHILES

Taking into account the limits of simplistic divisions that are often used with most extremophilic microorganisms, alkali-tolerant organisms can be defined as those that can grow at a pH of 9 or 10 but which have pH optima near neutrality (Krulwich, 1986; Krulwich and Guffanati, 1989; Wiegel, 1998). On the other hand, the term *alkaliphile* is used for microorganisms that grow optimally or very well at pH values above 9 but cannot grow or that grow only slowly at the near-neutral pH value of 6.5. Many different taxa are represented among the alkaliphiles, and some of these have been proposed as new taxa. Alkaliphiles can be isolated from normal environments such as garden soil, although viable counts of alkaliphiles are higher in samples from alkaline environments. The cell surface may play a key role in keeping the intracellular pH value in the range between 7 and 8.5, allowing alkaliphiles to thrive in alkaline environments, although adaptation mechanisms have not yet been clarified.

7.4.1. Alakaliphilic Ecosystems

Naturally extremely alkaline environments occur in “soda lakes,” where high evaporation rates in closed drainage basins occur. The water contains scarce amounts of Mg^{2+} and Ca^{2+} , and is nearly saturated with sodium salts, especially chloride, carbonate, and bicarbonate, with the pH generally around 10, due to high levels of sodium carbonate (Duckworth et al., 1996). Alkaline environments are also noted in pockets in ordinary soils where transient alkalinity is proposed due to various biological activities (Grant et al., 1990); indeed, alkaliphilic microorganisms have often been isolated from ordinary soils and other nonalkaline environments (Jones et al., 1998; Wiegel, 1998). A few natural habitats are hypersaline and alkaline, harboring a large group of haloalkaliphiles, especially from Lake Magadi in Kenya, the Wadi Natrun Lakes in Egypt (Imhoff and Trüper, 1977; Mesbah and Wiegel, 2008), and recently from soda lakes of the Kulunda Steppe in Altai, Russia (Sorokin and Muyzer, 2010). Many of these organisms are also thermotolerant to various degrees, making these lakes interesting sources of novel microorganisms (Jones et al., 1998; McGenity et al., 2000). Human-made alkaline environments include effluents from tanneries, paper mills, food- and textile-processing plants, calcium carbonate kilns, and detergents and other industrial processes. They all have in common the high levels of sodium (although it may be as low as 5% w/v) and concentrations of carbonate and bicarbonate which greatly exceed those of Mg^{2+} and Ca^{2+} . Thus, the Mg^{2+} and Ca^{2+} tend to precipitate as insoluble compounds with carbonate, leaving the excess anion to form sodium salts and counter the buffering effect of CO_2 . In addition, microbial processes such as ammonification and sulfate reduction led to the establishment of a stable, perpetually alkaline environment (Cavicchioli and Thomas, 2000).

7.4.2. Biotechnology of Acidophilic and Alkaliphilic Microorganisms

Much industrial interest in extreme acidophiles has been shown, with subsequent application having been successful in the area of biomining: the use of acidic bioprocesses to remove valuable metallic minerals from sulfide ores (Das et al., 1999). Other uses are envisioned: Acidophilic sulfate reducers may actually contribute net alkalinity to the sites and could be used for acid mine drainage remediation (Johnson, 1995; Castro and Moore, 2000; Sharma et al., 2011); the utilization of aliphatic compounds by some acidophiles, in combination with their tolerance of heavy metals, makes them candidates for bioremediation of acidic wastewaters contaminated with toxic organic compounds and heavy metals (Gemmell and Knowles, 2000). Most of the prokaryotes useful in these areas are extreme acidophiles, with $pH_{opt} < 3$ (Norris and Johnson, 1998), and are often subclassified based on their temperature-range preferences or their metabolic processes (i.e., heterotrophic vs. autotrophic, iron oxidizing vs. non-iron oxidizing).

Research and development has been focusing particularly on the potential use of mesophilic and psychrophilic acidophiles for biomining, such as tank leaching processes and heap leaching processes. Moreover, the application of moderately thermophilic and extremely thermophilic acidophiles for biomining and in the treatment of both refractory gold-bearing and base-metal mineral sulfide concentrates has also been investigated extensively (Johnson, 1995, 2001; Das et al., 1999; Castro and Moore, 2000; Dopson et al., 2004; Rawlings and Johnson, 2007; Johnson and Hallberg, 2008; Cardenas et al., 2010; Dopson, 2010). Some research activities regarded the problems of arsenic toxicity to

certain strains of moderately thermophilic bacteria when oxidizing both refractory gold and base-metal sulfide concentrates. Other potential applications of thermophilic acidophiles are usually related to the minerals industry and in allied disciplines, including treatment of metalliferous mine wastes, acid mine waters, and sulfurous gases.

The group of alkaliphilic microorganisms is another example of potential usefulness, with alkaline proteases and cellulases adapted for use in laundry and other detergents (Horikoshi, 1996), xylanases for use in the pulp and paper industry (Nakamura et al., 1993), and lipases in bioprocessing of lipids (Bornscheuer et al., 2002; Salameh and Wiegel, 2007). Enzymes of alkaliphiles are also used for dehairing of hides in leather tanning, making of food additives, and drug manufacture. One particularly important industrial application has been the production of cyclodextrin for use in foodstuffs, chemicals, and pharmaceuticals, using alkaline cyclomaltodextrin glucanotransferase (Horikoshi, 1996); moreover, other possible applications are being explored for enzymes from these organisms (Ahlawat et al., 2007; Asha Poorna and Prema, 2007; Singh et al., 2008).

7.5. HALOPHILES

Higher organisms can be divided between those whose cells require relatively low (i.e., 0 to 0.9% for humans—called “normal” or “physiological”) NaCl concentrations and those whose cells require elevated concentrations (i.e., marine fish do best at 3% NaCl, the salinity of ocean water and human tears). However, the very high salt concentrations in hypersaline lakes or soils, salterns, or other human-made environments, such as in salted foods, preclude the growth of most fish, invertebrate animals, plants, and even most prokaryotes, but there are microorganisms that thrive there. Like marine fish, mammals, and invertebrates, marine microorganisms must at least tolerate ocean water salinity. In contrast to halotolerant organisms, obligate halophiles must require NaCl concentrations higher than 3% w/v for optimal growth. The requirement for NaCl may be “slight” [most rapid growth at 2 to 5% (0.34 to 0.85 M) NaCl], “moderate” [most rapid growth at 5 to 20% (0.85 to 3.4 M) NaCl], or “extreme” [most rapid growth at 20 to 30% (3.4 to 5.1 M) NaCl] (Larsen, 1962). Other definitions have been published, including that true halophiles grow optimally at and above 10% NaCl.

7.5.1. NaCl-Enriched Ecosystems

The habitats that contain high salt concentrations are diverse (Fig. 7.6). Some authors feel that understanding the adaptations of terrestrial halophilic archaea may be important in detection of life on Mars, assuming similar types of salts and a carbon-based life form (Litchfield, 1998). Several of the hypersaline soda lakes (Lake Magadi in Kenya, the Wadi Natrun lakes in Egypt, and Mono Lake, Big Soda Lake, and Soap Lake in the western United States) are highly alkaline, with pH values of 9 to 11, while the Great Salt Lake and the Dead Sea have pH values around 7 (Ollivier et al., 1994). Unusual habitats have been found to be populated with halophiles, such as oil-field water in the North Sea (Lien et al., 1998), preserved salted foods (Kobayashi et al., 2000), and the nasal cavities of desert iguanas (Deutch, 1994; Lawson et al., 1996), although the ocean, hypersaline lakes of oceanic (thalassohaline) or nonoceanic (athalassohaline) origin, and solar salterns make up the predominant habitats (Antunes et al., 2008). Halotolerant or halophilic microorganisms

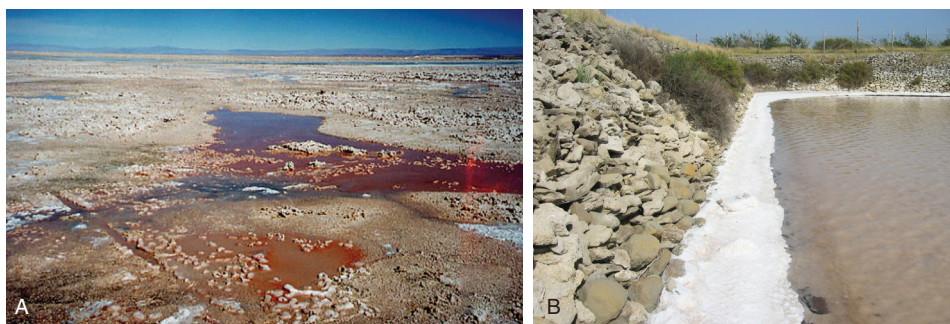


Figure 7.6. Two examples of ecosystems characterized by elevated NaCl concentrations: (A) Atacama desert (Chile); (B) Mediterranean solar salterns (Italy). (Courtesy of F. Canganella.) (See insert for color representation of the figure.)

have also been isolated from polar sea ice (Bowman et al., 1998) and from Antarctic rocks (Smith et al., 2000). Salinities in the various habitats can range from that of brackish waters to saturation, or from about 0.5% to 37% or more.

The predominant salts are often Na^+ and/or Cl^- , although Mg^{2+} or Ca^{2+} may be in abundance (except in the soda lakes; see Section 7.4.2), and all are usually noted in higher concentrations than in more moderate habitats. Sulfate is generally an important electron acceptor in these ecosystems, and salterns generally show precipitation of calcium compounds (CaSO_4 and CaCO_3) as well as NaCl, whereas soda lakes have carbonate precipitates of magnesium and calcium, with few of these cations in solution.

7.5.2. Biotechnology of Halophilic Microorganisms

Several practical applications are envisioned from the study of halophiles: better understanding of the salted and fermented foods technology, of the remediation of saline wastewater (Kargi and Dincer, 1998, 2000), and of the bioremediation of toxic compounds such as uranium (Francis et al., 2000), hydrocarbons (Bruns and Berthe-Corti, 2000; Mnif et al., 2009; McGenity, 2010), and pollutants (Le Borgne et al., 2008). Furthermore, production of biopolymers has been described for bacterial and archaeal halophilic microorganisms (Hezayen et al., 2000; Mata et al., 2008; Van-Thuoc et al., 2008; Quillaguamán et al., 2009).

7.6. PIEZOPHILES

The term *obligate*, or *extreme*, *piezophile* has been used for an organism incapable of growth at atmospheric pressure regardless of temperature, and the term *piezotolerant* has been applied to an organism that grows best at atmospheric pressure but can also grow at elevated pressures. One definition also states that extremely piezophilic bacteria are those unable to grow at pressures below 50 MPa, but able to grow well at 100 MPa (Kato et al., 1998).

There is a contradiction in the literature regarding definitions of pressure limits for microbial growth, due primarily to the complicating effects of temperature. The majority of piezophiles (barophiles) are also psychophilic, as the majority of the deep sea is cold



Figure 7.7. Deep-sea hydrothermal vent chimney. (Modified from *Focus* magazine.) (See *insert* for color representation of the figure.)

(<4°C). However, many prokaryotes isolated from hydrothermal vent environments are piezophilic (barophilic) as well as (hyper)thermophilic and mesophilic. To the latter belong the symbiotic chemolithotrophic bacteria of the tube-dwelling worm *Riftia pachyptila*, which has been taken as a symbol of deep-sea vent animal communities for many years (Figs. 7.7 and 7.8). These sulfur-oxidizing and carbon-fixing microorganisms are present in enormous numbers inside the animal's body, and they are capable of supplying most of the worm's metabolic needs, thanks to the fixation and incorporation of CO₂ (Cavanaugh et al., 1981; Robidart et al., 2008).

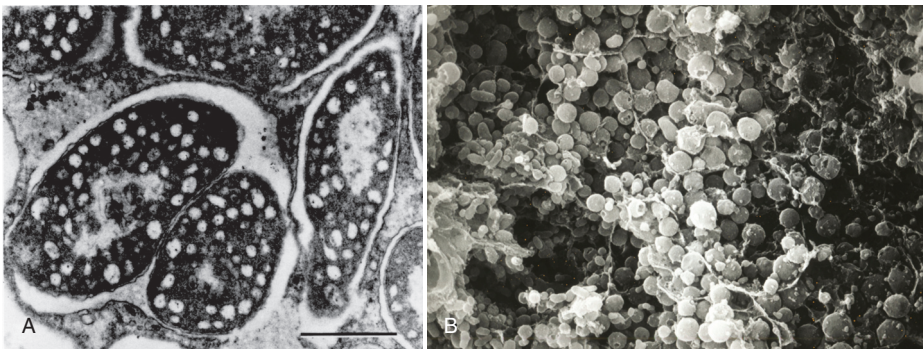


Figure 7.8. Syntrophic bacteria of *Riftia pachyptila*: (A) electronic transmission image; (B) electronic scan. Scale bar: 1 μ m. (Courtesy of C.M. Cavanaugh.)

7.6.1. Ecology of Piezophiles

Zobell and Morita (1957) isolated some bacteria from environments under extremely high pressure, but were partially successful. When better technology became available to explore the depths of the oceans and cultivate their microbial inhabitants, new life forms were discovered with unique adaptations to these habitats (Yayanos et al., 1982). While habitats in ocean depths have variable temperatures and pH's, all are under enormous pressure from the weight of the water column (up to 1100 atm in the deepest part of the Mariana Trench—Abe et al., 1999), and it was obvious that living organisms from those depths were tolerant of such pressures. Some of these microorganisms actually grow better (i.e., faster doubling times) under higher pressures than atmospheric, and some will not live and grow without pressures greater than or equal to 2.5 atm (Kato et al., 1996). These were referred to as barophilic, or now by the more appropriate term *piezophilic*; however, the majority of them are piezotolerant and only a few are (obligate) piezophiles.

7.6.2. Biotechnology of Piezophiles

Some bacteria are capable of living a high-pressure existence, and to do so they must have some peculiar features to sustain such a stringent environmental condition. High-pressure-adapted enzymes may be useful in high-pressure food processing, in breaking down toxic waste, and as substitutes for the harsh chemicals used in paper production.

Without doubt, pressure effects on protein structure and stability are important in various biological systems, including multimeric enzymes, ribosomes, cytoskeleton proteins, and proteins that act in signal transduction pathways; such interactions are thought to be sensitive to increasing pressure. Hydrophobic interactions, which have been implicated in stabilization of many thermophilic proteins at high temperature, also have a role in the stabilization of these proteins under elevated-pressure conditions.

From the viewpoint of industrial applications, enzyme stability is obviously important. So an understanding of the factors involved in increasing the stability of industrial products, as well as the factors to be considered in assessing the stability of newly designed proteins, may be fundamental for biotechnological processes. In this regard, the search for potential applications of piezophilic microorganisms is currently focused on biocatalytic processes, bioconversions, molecular biology, and food technology.

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THE ROLE OF EXTREMOPHILIC MICROORGANISMS AND THEIR BIOPRODUCTS IN FOOD PROCESSING AND PRODUCTION

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8.1. INTRODUCTION

The involvement of microorganisms in the production of processed food and drink has a long and well-established history, going back millennia. Microbial fermentation was used to make bread and beer, providing food and drink for diverse cultures in many parts of the world. The Egyptians discovered that if bread dough were allowed to stand for a few hours after mixing, the dough expanded and could be used to make bread with a lighter and spongier texture. Beer goes back 8000 years to ancient Iraq, and most civilizations around the world discovered methods of making fermented alcoholic drinks from locally available grains. The earliest evidence of wine from residues in pottery jars dates back to around the same time, in present-day Georgia. How this process was accomplished was, however, unknown until the nineteenth century, when the role of microorganisms was revealed (Hornsey, 2003). Cheese was invented in antiquity, when milk stored in bags made from ruminant stomachs was found to coagulate, due to the action of proteases from the stomach tissue on the curd proteins of milk (Fox and McSweeney, 2004).

These fermentative and hydrolytic processes all require the action of various enzymes. It was not until the nineteenth century that the role of enzymes was appreciated, and in the late nineteenth and early twentieth centuries, more and more enzyme-based production processes were developed, many related to food production (Poulsen and Buchholz, 2003). As the twentieth century continued, the nature and mechanisms of enzymatic action in fermentation and other processes became more fully understood. The advent in the 1960s

of commercially available glucoamylase, an enzyme that catalyzes the removal of glucose from starch, began the widespread large-scale application of purified enzymes for food processing and other applications. The molecular biology revolution of the 1980s made it possible to clone the genes encoding industrially useful enzymes and to manipulate their sequences to create genetically engineered enzymes with improved, and in some cases totally novel, properties.

The majority of the microorganisms used in food processing, and the enzymes derived from these, have been obtained from mesophiles. For many applications, these are adequate. However, for applications that require extremes of pH, temperature, or ionic strength, these enzymes may be unstable or inadequate, and entire microorganisms may not be viable. Hence, extremophiles, which thrive in environments that would be hostile for other organisms, provide a source of novel bioproducts that have existing and potential value in the food industry as well as in a range of other industries. The various categories of extremophilic microorganisms have been reviewed extensively elsewhere (Rothschild and Mancinelli, 2001; Pikuta et al., 2007; Canganella and Wiegel, 2011) and have traditionally been defined with respect to temperature, pH, salinity, hydrostatic pressure, and tolerance of ionizing radiation, as well as oxygen tension and chemical extremes. Some extremophiles are adapted to multiple categories of extreme conditions (Bowers et al., 2009).

Survival under these conditions has led to the development of a suite of physiological adaptations at a molecular level that allow these microorganisms to grow and thrive. As a result, biotechnologists are interested in exploiting novel molecules from extremophiles for a wide range of applications, including “green” chemistry, energy generation, medicine, and as discussed in this chapter, the food industry. Thermophilic microorganisms produce enzymes that are stable at the high temperatures required for some processing applications, and psychrophiles and psychrotrophs produce enzymes with relatively high activity in refrigerated or other cold environments. Other extremophiles, particularly halophiles, produce compatible solutes and other molecules that may be used in food processing.

Cold-adapted microorganisms also have a role in spoilage of refrigerated foods, and some are pathogens of farmed fish species that live in cold water. Osmophilic bacteria and fungi can cause spoilage in foods containing sugar, such as fruit juices, jams, and honey; xerophiles spoil dried foods; and halophiles are found in salted foods. Radiore-sistant microorganisms are not inactivated by irradiation, which is used to sterilize food and facilitate preservation. In short, extremophiles play a role in many aspects of food production, spoilage, and preservation, and the exploitation of their bioproducts is only in its early stages.

8.2. ENZYMES FROM EXTREMOPHILES IN FOOD PROCESSING

In 2009, food enzymes constituted the largest market share of industrial enzymes, although the fuel ethanol enzyme market is growing rapidly. The three major world suppliers of industrial enzymes are Novozymes A/S (Denmark); Genencor International Inc. (the United States), and DSM N.V. (the Netherlands), with the largest of these being Novozymes (Market Research News, 2011). However, there are many other companies worldwide that supply industrial enzymes, and Indian and Chinese companies are likely to take an increased share of the market (Indian Enzymes Market report, 2011; Market Research Reports, 2010).

Food enzymes are used for many reasons: They are invaluable in milk processing, cheese making, juice making, vegetable processing, brewing, and baking. They are also used in animal feed production. Enzymes are specific for particular reactions and work at relatively mild values of pH, temperature, pressure, and ionic strength, while remaining highly active. They are also biodegradable and reusable in multiple production cycles when immobilized in enzyme reactors. With the interest in green chemistry and the need to minimize the environmental impact of industrial processes, enzyme technology is likely to expand in coming years. The disadvantages of enzymes include their high cost (particularly when isolated from natural sources, e.g., plants, animals, and slow-growing microorganisms), fragility, contamination with pathogens, and in some cases, narrow substrate specificity. However, the advent of recombinant DNA technology in the 1980s has enabled the production of industrial enzymes at relatively low cost and, crucially, their modification to facilitate industrial processes (Hjort, 2007). Enzymes can also be immobilized onto solid supports, allowing reuse of the enzyme and facilitating the recovery of the product without contamination with the enzyme (Walsh, 2007).

Enzyme discovery has been facilitated by functional screening of pure cultures of microorganisms and metagenomic approaches. Metagenomics involves the screening of clone libraries of gene fragments and amplifying with primers that identify a particular enzyme or family of enzymes. The genes can be derived from almost any source in nature, including the gut microflora, soil, and ocean water (Daniel, 2005; Ferrer et al., 2007; Uchiyama and Miyazaki, 2009; Fernández-Arrojo et al., 2010; Grant and Heaphy, 2010). Extremozymes (i.e., enzymes derived from extremophiles) can be identified by functional screens of cultures of extremophilic microorganisms, but this does not resolve the issue of unculturable (i.e., microorganisms that will not grow in the laboratory) or problems with culturable but difficult-to-grow microorganisms. Metagenomic techniques in which DNA fragments are cloned into mesophilic hosts (e.g., *Escherichia coli*) provide a way of accessing these valuable biocatalysts (Steele et al., 2009; Lee et al., 2010; Kennedy et al., 2011). The availability of high-throughput techniques, molecular biology, and computer-assisted design of proteins has also facilitated the engineering of enzymes to enhance their catalytic properties or stability (Bershtein and Tawfik, 2008).

Some processes require acidic or basic pH for optimal operation, so extremozymes that can withstand these conditions (which can sometimes be obtained from multi-extremophiles, e.g., thermoacidophiles) are actively sought in nature or by introducing mutations. Hyperthermophiles (growth temperature 80 to 113°C) are widely distributed among the archaea (which include thermoacidophilic species among the genera *Acidianus*, *Ferroplasma*, *Picrophilus*, *Sulfolobus*, and *Thermophilus* that grow aerobically from 60 to 90°C and at pH 0.7 to 4.0). Thermoalkaliphiles also exist: for example, three strains of *Thermococcus* grow optimally at pH 8.5 to 9 and 85°C (Egorova and Antranikian, 2005). The remarkable properties of these microorganisms make them ideal sources of exceptionally robust enzymes.

With respect to the food industry, thermophiles and psychrophiles are the most important sources of enzymes. This is because for some processes, operation at high temperature minimizes problems with microbial contamination, particularly for continuous processes without regular disinfection, and some reactions are more favorable at high temperature (e.g., the conversion of glucose to fructose) (Fernandes, 2010). Other processes are best suited to catalysis by cold-adapted enzymes, which can be derived from prokaryotes or eukaryotes (e.g., cold-water fish and crustaceans). The processes that could employ

cold-adapted enzymes include milk processing, clarification of fruit juices with pectinases, brewing, or oligosaccharide production (Fernandes, 2010).

The application of any enzyme in the food industry, irrespective of source, is subject to national laws and regulations to ensure consumer safety. In Europe, there are food safety laws that are harmonized throughout the European Union and others that are solely national (Praaning et al., 2003). In the United States, the use of enzymes in food processing is regulated by the Food and Drug Administration (FDA). Enzymes are regarded as food additives and have to go through rigorous toxicological testing before they are approved as “generally recognized as safe” (Whitaker, 2003). These processes take time, and an extremozyme may take years to be tested and purified to the extent that it is approved for food use.

8.2.1. Enzymes from Thermophiles

Thermophilic enzymes are characterized by increased surface charge, increased ionic interactions and hydrogen bonds, increased hydrophobicity, smaller surface loops, and decreased flexibility, all of which contribute to their stability at high temperatures (Sternier and Liebl, 2001; Trivedi et al., 2006). The majority of thermophilic enzymes used in food processing are hydrolases. These include carbohydrate-processing enzymes (e.g., α -amylase and glucoamylases) that are used in starch saccharification as well as in baking and brewing. Lipases and proteases are the other most widely used hydrolases in the food and drink industries. Transferases (e.g., fructosyltransferase) are used, as are isomerases (e.g., glucose isomerase) in starch processing and sugar production.

Carbohydrate-Degrading Enzymes. Starch is a polysaccharide comprised of α -glucose monomers, linked by α -(1,4)- and α -(1,6)-glycosidic bonds. It is used in the food industry as a thickener, binder, and gelling agent and also as a source of sugar syrups used to make confectionery and sugary drinks. It has been estimated that 10 to 15% of the world enzyme market consists of enzymes used in starch processing, but variations in process conditions, low thermostability, and instability at pH extremes make the use of thermostable and acid-stable enzymes desirable (Satyanarayana et al., 2006). For food-processing applications, α -amylase is obtained by controlled fermentation of *Bacillus stearothermophilus*, and *B. subtilis* containing a *B. megaterium* or *B. stearothermophilus* gene. It is used to convert starch to dextrins, which are further hydrolyzed by glucoamylase to yield glucose feedstock for making corn syrup, ethanol, or alcohol in beverages. The enzyme can also be added to bread dough to provide the yeast with a continuous supply of sugar for fermentation and carbon dioxide production, and to improve the softness and moistness of bread, lengthening its shelf life (Fernandes, 2010).

The process of starch hydrolysis requires gelatinization to dissolve the starch granules (i.e., liquefaction); thermostable α -amylase is used to hydrolyze the α -(1,4)-linkages to dextrins at high temperature (95°C), followed by a further hydrolytic step to produce glucose and maltose. Because of this, the starch industry requires highly thermostable α -amylases (Prakash and Jaiswal, 2010). Many of these are Ca^{2+} dependent, although that of the extreme thermophile *B. thermooleovorans* NP54 is Ca^{2+} independent, which is advantageous, as continuous Ca^{2+} addition to the reaction mixture is not required (Malhotra et al., 2000). Some α -amylases are also unstable at low pH. While *Bacillus* spp. are the most widely used sources of thermostable α -amylases, *Pyrococcus* spp., *Rhodothermus marinus*,

Thermococcus spp., *Thermotoga maritima*, and *Thermus filiformis* all produce this enzyme (Prakash and Jaiswal, 2010). Commercially available thermophilic α -amylases include the Novozymes product Thermamyl Brew Q from *B. licheniformis*. Some α -amylases have also been engineered to improve their properties (e.g., that of *Thermus* sp. IM6501, which was modified to improve its thermostability at 80°C) (Kim et al., 2003).

Pullulanases are amylolytic exoenzymes that degrade pullulan, which is a chain of maltotriose units linked by (1,6)-glycosidic bonds. These enzymes are used mostly in conjunction with other enzymes (e.g., glucoamylases) as part of the saccharification process that converts starch into glucose, and sometimes in baking. The commercially available preparations are generally produced by *Klebsiella* and *Bacillus* spp., as well as the fungus *Trichoderma* (Doman-Pytka and Bardowski, 2004). However, some thermophiles also produce pullulanases: *Thermoanaerobacterium thermosaccharoliticum* and the thermoalkaliphile *Anaerobranca gottschalkii*, which produces a heat and alkali-stable type I pullulanase (Bertoldo et al., 2004), *Rhodothermus marinus* (Gomes et al., 2003), and *Geobacillus thermoleovorans* NP33 (Satyanarayana et al., 2004).

Glucoamylases [Kumar and Satyanarayana (2009) list a wide range of their microbial sources and properties] complete the final step in the production of glucose. They are inactivated at the high temperatures used for the initial step in starch degradation, and the mixture has to be cooled to 60°C to allow them to release glucose (Fernández-Arrojo et al., 2010). They are second only to proteases in terms of their sales among industrial enzymes. The demand for thermostable glucoamylases has led to their discovery in a range of thermophilic organisms: for example, the soil fungus *Chaetomium thermophilum* (Chen et al., 2005), a thermophilic *Bacillus* sp. (Gill and Kaur, 2004), *Clostridium* sp. 0005 (Ohnishi et al., 1992), *Thermomucor indicae-sensuatae* (Satyanarayana et al., 2004), *Thermoanaerobacter tengcongensis* (Zheng et al., 2010) and the archaeal species *Sulfolobus solfataricus* (Kim et al., 2004), *Thermoplasma acidophilum* (Dock et al., 2008), *Picrophilus torridus*, and *Picrophilus oshimae*. The latter three archaeal species produce glucoamylases that are optimally active at pH 2 and 90°C, with catalytic activity remaining at pH values as low as 0.5 and at 100°C (Egorova and Antranikian, 2005).

Glucose isomerase (xylose isomerase) carries out the isomerization of hexose, pentose, and tetrose sugars, making it extremely useful for the production of high-fructose corn syrups as well as for the production of ethanol from hemicelluloses. It is produced commercially from various mesophilic microorganisms (Deshpande and Rao, 2006). The conversion of glucose to fructose at high temperatures may be optimized in various ways, one of which is improving the enzyme's thermostability to allow process operation at higher temperatures. Hyperthermophiles (e.g., *Thermotoga* spp.) have been investigated as sources of thermostable glucose isomerases, and these were found to be suitable biocatalysts at high temperature provided that they were immobilized and substrate lability was minimized (Bandlish et al., 2002). The thermophile *Fervidobacterium godwanense* also produces a xylose isomerase with optimal activity at 70°C (Kluskens et al., 2010) and higher catalytic activity than the *Thermotoga maritima* enzyme at 85°C. The conversion of D-galactose into D-tagatose has also been accomplished by production of a thermostable L-arabinose isomerase from *Thermoanaerobacter mathranii*, which was immobilised and used to produce D-tagatose at 65°C, and combined with a commercially available glucose isomerase into a sweetening mixture (Jørgensen et al., 2004).

Invertases are among the most widely used enzymes in the food industry, being used widely in the production of jams, confectionery, and preserves. They remove the terminal

nonreducing L,D-fructofuranoside, and their preferred substrate is sucrose, although some can hydrolyze rhamnose and stachyose (Gracida-Rodríguez et al., 2006). While mesophilic yeasts are the commonest source, it has been found in thermophilic microorganisms. For example, *Thermotoga neapolitana* DSM 4359 produces an invertase with an optimum temperature of 85°C at pH 6 (Dipasquale et al., 2009).

Many bacteria and fungi produce xylanases as part of the suite of enzymes that allows them to invade plants and degrade xylan from plant cell walls. Their most common application is in the paper and pulp processing industry, but they also have food applications. In particular, they can be added to animal feeds in combination with β -(1,3)- and (1,4)-glucanases to enhance their digestibility (Mathlouthi, 2003), and they can also be added to wheat flour to enhance its quality (Haros et al., 2002). Jiang et al. (2005) found that a thermostable xylanase, XynB, from *Thermotoga maritima* when added to wheat flour dough retarded bread staling, and when used for making partially frozen baked bread led to a significant increase in dough extensibility, swelling, and a decrease in dough resistance to deformation (Jiang et al., 2008). *Pyrodictium abyssi*, a thermophilic archaeon, produces a xylanase with an optimum temperature of 110°C, one of the highest-temperature optima reported for a xylanase (Carvalho Andrade et al., 2001), which may find commercial use in the future.

Arabinofuranosidases are also part of xylanolytic systems, and these enzymes can be used to enhance the aroma of wines and fruit juice, as well as to produce arabinose for the food industry in general. In one study, the thermophilic eubacterium *Rhodothermus marinus*, which also produces a xylanase, was found to produce a thermostable α -L-arabinofuranosidase, and its production conditions were optimized (Gomes et al., 2000).

The exoskeletons of insects and marine crustaceans, and the cell walls of some fungi, are composed of chitin, which is an unbranched homopolymer of (1,4)-linked *N*-acetyl- α -glucosamine. This material is degraded by chitinases, which are produced by many organisms, including insects, plants, bacteria, and fungi as well as vertebrates, in which chitinases appear to function in innate immunity. The seafood-processing industry generates large quantities of chitinous shells as a by-product. These can be converted to chitosan, which has pharmaceutical and cosmetic applications, and also glucosamine, a food supplement. Chitosan has the potential to become a major ingredient of functional foods, but has not yet gained wide acceptance in the United States, where sales were estimated at about \$20 million per year. Chitosan has also been tested as a food preservative for fruits and vegetables, preventing fungal and bacterial growth (for a review of applications, see Hayes et al., 2008). Thermostable chitinases from extremophiles could potentially be extremely valuable for chitin-processing applications at high temperatures.

To carry out the degradation of chitin at temperatures where chitinolytic enzyme activity is high, thermostable chitinases from thermophilic microorganisms have been sought from a number of sources. They have been isolated from the hyperthermophilic archaea *Pyrococcus furiosus* (Gao et al., 2003), *Thermococcus chitonophagus* (Andronopoulou and Vorgias, 2003), and *T. kodakarensis* (Tanaka et al., 2003), the thermophilic eubacteria *Clostridium thermocellum* (Zverlov et al., 2002), *Bacillus* spp. (Takayanagi et al., 1991), and the thermophilic fungi *Thermoascus aurantiacus* var. *levisporus* and *Chaetomium thermophilum* (Li et al., 2010).

β -Galactosidases are produced by a range of microorganisms and are used to hydrolyze lactose in milk. A recombinant thermostable β -galactosidase from *Bacillus stearothermophilus* immobilized onto chitosan gave rise to a greater than 80% hydrolysis of

lactose in milk after 2 h in a packed-bed reactor. This preparation thus shows promise as a method of producing lactose-hydrolyzed milk (Di Serio et al., 2003). An immobilized enzyme produced from a *Thermus* sp. led to a greater than 99% hydrolysis of lactose, even at 70°C (Pessela et al., 2003). Some of these enzymes from thermophiles also have transglycosylation activity. After lactose hydrolysis, the galactose liberated may be transferred onto disaccharide or trisaccharide acceptors, generating galacto-oligosaccharides (Nakkharat et al., 2006). In food processing, β -mannanases are used to macerate fruit and vegetables, clarify juices, extract oil from legume seeds, and reduce viscosity of coffee extracts in making instant coffee and in making wine and beer (Huerta-Ochoa et al., 2006). Most commercially used β -mannanases are from mesophiles, but they are also present in some thermophiles (e.g., *Bacillus stearothermophilus*) (Ethier et al., 1998) as well as an extreme thermophile *Dictyoglomus thermophilum* Rt46B.1. The latter enzyme had a temperature optimum of 80°C, which would make it suitable for use at high temperatures (Gibbs et al., 1999).

Proteases. The large-scale use of proteases occurs in many industries, particularly the detergent industry, in which they are added to household detergents. Alkaline serine proteases (subtilisins) from mesophilic *Bacillus* spp. are commonly used. These enzymes are quite stable at about 60°C and are active at pH 9 to 11 (Kristjánsson and Ásgeirsson, 2003). Highly thermostable proteases could be used to hydrolyze protein wastes at high temperatures and for making protein hydrolysates, as well as helping to prevent the growth of spoilage bacteria and various pathogens during processing. Food-grade proteases are commercially available from many different companies, but in general these are obtained from mesophilic bacteria, fungi, plant, and animal sources (Sumantha et al., 2006). Thermolysin, a thermophilic neutral metalloprotease, is produced by *Bacillus thermoproteolyticus*, a gram-positive thermophile. This enzyme is remarkably thermostable, retaining 40% of its activity after 1 h at 80°C (Sumantha et al., 2006). It can hydrolyze peptide bonds on the amino terminal side of hydrophobic residues (e.g., leucine, phenylalanine, isoleucine, and valine). One of its applications is the synthesis of aspartame, which is made from aspartic acid and phenylalanine. The enzyme recognizes only the L-isomer of phenylalanine, and the β -carboxyl group of the aspartate does not require protection, ensuring that only one isomer is made (Walsh, 2007). Thermolysin is also used for the production of bioactive peptides from milk and whey proteins (Ortiz-Chao and Jauregi, 2007).

Laccases. These enzymes are *p*-diphenol:dioxygen oxidoreductases, which are abundant in white-rot fungi. In nature, they degrade lignin and compounds with similar structures (e.g., polyaromatic hydrocarbons and a range of other phenolic substrates). As a result, they are commonly used to enhance or modify the color and appearance of various foods and beverages (e.g., beer and wine) (Osma et al., 2010). There are some thermophilic laccases, and the most widely used is that from the thermophilic fungus *Myceliophthora thermophila*, the gene for which is cloned into *Aspergillus oryzae* for production (Berka et al., 1997). This enzyme is sold as a preparation called Flavourstar, manufactured by Novozymes A/S, and is used to prevent off-flavor compounds (e.g., *trans*-2-nonenal) from forming when brewing beer (Food and Agriculture Organization of the United Nations, 2004).

Lipases. Lipases are used extensively in food production and processing (Aravindan et al., 2007; Houde et al., 2004), but most of these are derived from nonextremophilic

sources, including bacteria, fungi, and mammals (e.g., porcine pancreatic lipase). The dairy industry uses lipases for the hydrolysis of milk fats, acceleration of cheese ripening, and flavor development in cheese, as the differential release of long- or short-chain fatty acids imparts different flavors. Microbial lipases can be used as part of a substitute for mammalian rennet in the manufacture of some cheeses. Other applications for lipases include flavor development in meat, fish, and other foods, improvement of aroma in alcoholic beverages, formation of cocoa butter, and manufacture of pet foods. There is considerable interest in thermostable lipases such as those obtained from *Thermus aquaticus*, *T. flavus*, and *T. thermophilus* (the latter two are commercially available from Fluka/Sigma-Aldrich). Operation of industrial processes at elevated temperatures provides advantages: for example, a decrease in the risk of contamination, high mass transfer, and enhanced solubility of lipid substrates, as discussed by Kademi et al. (2006). *Rhizomucor miehei*, which grows optimally at 45°C and can grow up to approximately 58°C, produces a lipase sold by Novozymes under the trade name Palatase. This enzyme was developed for use in the dairy industry, in particular for flavor enhancement in cheese. Palatase has also been combined with a lipase preparation, Flavourzyme, to produce highly flavored cheese-like hydrolysates from milk (Brindisi et al., 2001). *Thermomyces lanuginosus* produces an *sn*-1,3-specific lipase that has been cloned in *Aspergillus oryzae* and is sold by Novozymes under the trade name Noopazyme. This enzyme is used to improve the quality of oriental noodles made from low-quality flour, and pasta from nondurum flour (Houde et al., 2004).

8.2.2. Enzymes from Cold-Adapted Organisms

Cold-adapted organisms are usually classified as psychrophiles or psychrotrophs (psychrotolerants). According to the definition of Morita (1975), psychrophiles have an optimum growth temperature of $\leq 15^{\circ}\text{C}$, with a maximum growth temperature of $\sim 20^{\circ}\text{C}$, whereas psychrotrophs can grow at low temperatures but have maximum growth temperatures of 20°C or above. Cold-adapted microorganisms, and indeed cold-adapted animals, are widely distributed, as most of Earth's biosphere is cold. These permanently cold environments vary from deep ocean waters, where the majority of the oceans are at temperatures of -5°C , to alpine environments and polar regions. Many food-spoilage microorganisms are psychrotrophs and can grow in refrigerated food, and some pathogens of cold-water fish are psychrotrophic or psychrophilic. In general, enzymes produced by psychrophiles compensate for the low kinetic energy available at low temperatures by having a more flexible structure than those of mesophiles and thermophiles. They show a reduction in activation enthalpy and have more negative values of activation entropy than those of their mesophilic and thermophilic counterparts (Siddiqui and Cavicchioli, 2006; Cavicchioli et al., 2011).

The structural characteristics of cold-adapted enzymes are in contrast to those of thermophiles and mesophiles, which are more stable at higher temperatures. Psychrophilic enzymes are characterized by relatively high activity at low temperatures and optimize their values for k_{cat} at the expense of K_m , possessing higher flexibility coupled with a less stable active site (Feller, 2010). They show increases in surface hydrophobicity with decreases in core hydrophobicity; decreases in weak electrostatic interactions; fewer proline residues in loops, and more loops; weak binding of stabilizing ions; an excess of negative or positive charges at the surface, which may improve interactions with the solvent; and fewer salt bridges on the molecule's surface. The extent and nature of these adaptations varies from protein to protein, and each protein "solves" the stability problem in a different manner.

However, in general, all cold-adapted enzymes share the characteristic of higher specific activity at lower temperatures. For many psychrophilic enzymes, the active site is larger and more easily accessible to ligands, allowing them to accommodate substrates at a lower energy cost and lowering the activation energy, but this is counterbalanced by increased thermolability as temperatures increase.

These properties, along with the ability of some enzymes to function in organic solvents (Siddiqui and Cavicchioli, 2006) make cold-adapted enzymes a perfect choice for certain applications. These include where formation of side products due to elevated temperatures is undesirable, the substrates of the reaction are heat labile, and the enzyme needs to be removed after the reaction is complete. The thermolability of these enzymes ensures that they can be inactivated by heating the mixture. Several recent reviews discuss the contribution of enzymes from psychrophiles to biotechnological applications (Cavicchioli and Siddiqui, 2006; Margesin and Feller, 2010; Cavicchioli et al., 2011). Applications of psychrophile biocatalysts are also covered in more general reviews on extremophile enzymes, such as those of Gomes and Steiner (2004), Kumar et al. (2011), and Trincone (2010), who deal specifically with biocatalysts from marine environments. The discovery of these enzymes came about, in many cases, through bioprospecting in cold environments, particularly the antarctic. Two reports of the United Nations University Institute of Advanced Studies describe bioprospecting activities in the Antarctic (Lohan and Johnston, 2005) and the Arctic (Leary, 2008). This institution has launched an online searchable database called Bioprospector (<http://bioprospector.org/bioprospector/antarctica/home.action>), which provides information about patents, commercial products, and companies that market or study bioproducts from this region.

The use of cold-adapted enzymes in food processing and in biotechnology in general is not as advanced as that of their thermophilic counterparts. However, there are some established applications for psychrophilic enzymes, as well as potential applications, which are listed by Cavicchioli and Siddiqui (2006). Processing of milk and dairy products is one area where cold-adapted β -galactosidases may find wide application (for a review of β -galactosidase applications, production, and sources, see Oliveira et al., 2011). Due to the inability of adults to synthesize intestinal lactase, lactose intolerance is widespread and causes digestive discomfort due to fermentation of lactose by the intestinal microflora. Several groups have identified and characterized cold-active β -galactosidases, and one from *Pseudoalteromonas haloplanktis* has been patented (U.S. Patent 6727084; WO 0104276; Hoyoux et al., 2001). This enzyme has stable activity at 4°C but is inactivated by pasteurization. Other applications of this enzyme include production of milk powders, for lactose hydrolysis in whey to produce syrup for making ice cream, in confectionery and baked goods, and for the production of whey for animal feed and the manufacture of fermented whey products. The Belgian company Nutrilab SV is developing the application of this enzyme in the production of D-tagatose, a sugar used as a high-value sweetener with a low glycemic index (http://reflexions.ulg.ac.be/cms/c_30824/l-enzyme-qui-produit-du-sucre). Several other cold-active β -galactosidases have been identified and characterized; these include those from *Arthrobacter psychrolactophilus* strain F2 (Nakagawa et al., 2007); *Arthrobacter* sp. 20B (Białkowska et al., 2009); and the psychrotolerant yeast *Guehomyces pullulans* 17-1 (Song et al., 2010). Some of these enzymes may be used in lactose hydrolysis in the future.

Catalase is used in making some types of cheese, and it is endogenous in milk. Its antimicrobial properties remove the requirement for pasteurization, which could affect

enzymes and other compounds essential for flavor development in the cheese. However, hydrogen peroxide also inhibits some of the enzymes required for cheese production, so it is removed by adding catalase to convert it to water and oxygen. Cold-adapted catalases such as those from enzymes from *Bacillus* strain N2a (Wang et al., 2008) could potentially be used in this process to remove H₂O₂ more rapidly at lower temperatures than those used at present.

A cold-active xylanase is used in baking to improve bread quality. *Pseudoalteromonas haloplanktis* TAH3a produces a novel family 8 xylanase (Collins et al., 2002; Van Petegem et al., 2003). This enzyme was evaluated in baking Belgian hard rolls and Argentinean breads and was found to increase bread loaf volume (Collins et al., 2006). Lower doses of this enzyme (as well as two other psychrophilic xylanases) were required to reach optimal bread volumes compared to other xylanases (Dornez et al., 2011). This product has been patented (US/2008/0020088, EP1723229, WO/2005/087916) and is sold by the Belgian company Puratos Naamloze Vennootschap.

Pectinases comprise a group of at least seven different enzymatic activities that are used to break down pectin, a structural polysaccharide found in the primary cell wall and middle lamella of vegetables and fruits. As a result, these enzymes—derived from many different sources—have been used in wine and juice processing for at least 70 years (Favela-Torres et al., 2006). Plants and a wide range of microbes produce these enzymes, but commercially, *Aspergillus* spp. and other fungi are the most widespread sources of these enzymes. Addition of these enzymes to fruit can increase juice yields, accelerate juice clarification, and promote the release (or breakdown) of pigments and flavor-adding components. Due to this widespread application, the annual worldwide use of these enzymes is in excess of 7×10^6 tons (Favela-Torres et al., 2006). For some food and drink applications (e.g., wine making), low temperatures (e.g., 10 to 15°C) are essential, because this increases the production and retention of volatile compounds, improving the aroma of wine. As a result, pectinolytic enzymes that can work in this temperature range are essential for extraction and clarification of grape juice.

One recent study of psychrotolerant pectinolytic microorganisms from Argentina has shown that cold-active pectinases, with good activity at temperatures as low as 3°C, were produced by *Bacillus* spp. SC-G and SC-H and also from two yeast strains isolated from grapes (Cabeza et al., 2009). Pectate lyase genes have been cloned and the enzyme over-expressed from the marine antarctic bacterium *Pseudomonas haloplanktis* strain ANT/505 was pectinolytic toward citrus pectin agar (Truong et al., 2001), with optimal activity at 30°C and a 2-min half-life at 40°C. The psychrophilic yeast *Mrakia frigida* also produces a cold-active pectate lyase with optimal activity at 30°C (Margesin et al., 2005). Birgisson et al. (2003) also found eight strains of cold-adapted yeasts, including *Cryptococcus* and *Cytophobasidium* spp. that produce cold-active polygalacturonidases. Although these enzymes are not yet commercially available, pectin-degrading enzymes from these or other cold-adapted microbes are likely to be used for wine and juice processing in the future.

Cold-active lipases are produced by bacteria and fungi growing in permanently cold habitats and also by microorganisms that grow in refrigerated milk and food samples (Joseph et al., 2007, 2008). These enzymes are largely extracellular and their production is strongly influenced by such environmental factors as temperature, pH, nitrogen and carbon sources, the presence of inducers (e.g., lipid substrates), and dissolved oxygen. *Candida antarctica*, a psychrotroph that can grow at 30°C (Adamczak, 2003) produces two lipases, A and B. Lipase B is available in immobilized form under the trade name Novozym 435

from Novozymes (and also from Sigma-Aldrich). Novozymes also produce another lipase from the same microorganism, under the trade name Novozym CALB L. This enzyme, along with lipases from *Candida cylindracea* AY30, *Pseudomonas* spp., and *Geotrichum candidum* have been used to make lipophilic antioxidants for use in sunflower oil (Buisman et al., 1998; Pandey et al., 1999), and *Pseudomonas* strain P38 lipase has been used to make a flavoring compound, butyl caprylate (Tan et al., 1996).

A great deal of interest in psychrophilic proteases has centered on their application in laundry detergents, which aid energy conservation by enabling washing at low to moderate temperatures. Cold-active subtilisins isolated from antarctic *Bacillus* spp. have been characterized (Davail et al., 1994), and these may also have applications in food processing. However, cold-adapted proteases have also been employed and are being investigated as tools for the food industry, although their purification and structural determination may be problematic, due to their thermolability and higher autolytic degradation than those of proteases from other sources. Gudmundsdóttir and Bjarnason (2007) reviewed the applications of cold-adapted proteases in the food industry, with particular reference to cold-adapted enzymes from Atlantic cold-water fish (e.g., trypsin, chymotrypsin and serine collagenase from the Atlantic cod). Cold-active proteases are used in processing caviar (releasing roe from roe sacks), de-skinning fish fillets or squid mantle, and removing the membranes from cod liver or swimbladder (Kristjánsson and Ásgiersson, 2003).

Another application of psychrophile-derived proteins, albeit without enzymatic activity, is that of antifreeze proteins. Many of these are small glycoproteins first found in fish in polar waters (Fletcher et al., 2001) that prevent the growth of ice crystals in cells and body fluids of these animals, which live in water at temperatures below the freezing point. The gene of an antifreeze protein from the ocean pout, which is found in northern oceans, has been cloned and expressed in *Saccharomyces cerevisiae*. Unilever now includes this recombinant protein, described as ice-structuring protein, in some of its ice-cream brands sold in the United States, Australia, and New Zealand, although not in Europe. It controls ice recrystallization following freeze–thaw cycles, which would adversely affect the taste and quality (Margesin and Feller, 2010).

8.2.3. Molecules from Halophiles

Halophilic microorganisms are adapted to moderate and high salt concentrations and comprise members of all three domains of life. Oren (2006) defines halophiles as microorganisms that grow optimally at Na concentrations in excess of 0.2 M. Many marine microorganisms are slightly halophilic (growing at approximately 3.5% v/v NaCl); moderate halophiles grow optimally at 3 to 15% NaCl, whereas the extreme halophiles, many of which are archaea, grow optimally at Na concentrations in excess of 10% w/v (1.7 M), and some organisms can grow in saturated salt solutions (30% w/v). Extremely salty environments with such salt concentrations are home to the Halobacteriaceae, which are archaea, and some bacteria, including the red extreme halophile *Salinobacter ruber*. To provide osmotic equilibrium in this high-salt environment, halophilic microbes accumulate compatible solutes (Shivanand and Mugeraya, 2011). These are low-molecular-mass compounds (e.g., sugars, alcohols, amino acids, betaines, and ectoines). The extremely halophilic Halobacteriaceae accumulate K^+ in the cytosol to counterbalance the high salt concentration in the environment.

Oren (2010) has reviewed the industrial and biotechnological applications of halophiles, including their growth in fermented foods. Some traditionally fermented foods, particularly those from some Asian countries, require the addition of large quantities of salt. These include *jeotgal*, a fermented seafood from Korea, *fugunoko nukazuke* from Japan, and the Thai anchovy fish sauce *nam-pla*. To make *nam-pla*, two parts of fish are added to one part of marine salts, covered with concentrated brine, and allowed to ferment for about a year. From this mixture, novel halophiles have been isolated [e.g., *Lentibacillus halophilus* (Tanasupawat et al., 2006), *Halococcus thailandensis* (Namwong et al., 2007), *Natrinema gari* (Tapingkae et al., 2008)], although the role that these species play in the development of the dish is unclear. *Jeotgal* has also been found to contain halophilic archaea (Roh et al., 2007). *Tetragenococcus halophila*, a halophilic lactic acid bacterium that can grow in up to 4 M NaCl, grows in both Japanese and Chinese soy sauces during fermentation, but there are different strains of this bacterium, and considerable heterogeneity in substrate preference, growth, and acidification characteristics was found (Röling and van Verseveld, 1996). The highly salt-tolerant psychrophile *Chromohalobacter beijerinckii* has been found in salted food and produces biogenic amines (Beutling et al., 2009).

To date there has been relatively little demand for enzymes from halophiles in the food industry, but halophiles have a place as sources of compatible solutes and other compounds (e.g., β -carotene). The green algae *Dunaliella salina* and *Dunaliella bardawil* are major sources of β -carotene, one of the main halophile by-products. *Dunaliella* spp. are unicellular photosynthetic motile algae lacking a rigid cell wall, and they are isolated from aquatic saline habitats (e.g., sea and inland salt lakes containing from 0.5 to 5.0 M NaCl). *D. salina* accumulates large quantities of β -carotene as well as other carotenoids in its chloroplast as a protectant against light-induced damage, and the organism can contain up to 14% dry weight of this substance (Aasen et al., 1969). The molecule can be in a 9-*cis* and all-*trans* form as well as a 13-*cis* and 15-*cis* form. *Dunaliella* β -carotene is sold as a food supplement, sometimes as part of a mixture with other nutrients. It can also be added to foods as a coloring agent and can be added to animal foods as a source of vitamin A or as an enhancer of yolk color in vitamin A-deficient chicken (Raja et al., 2007). *D. salina* is also a source of lipids rich in polyunsaturated fatty acids (Abd El-Baky, 2004) that can be added to food products or pharmaceuticals. Recently, Pérez et al. (2011) described the isolation of a halophilic lipase from *Marinobacter lipolyticus* SM19, which was found to be highly efficient in producing eicosapentaenoic acid (EPA), an omega-3 polyunsaturated fatty acid, from fish oil. This enzyme has potential for producing EPA for food enrichment.

The main biotechnological application of halophiles to date, although not as yet to a major extent in food and nutrition, is as sources of compatible solutes. The best known of these to date is ectoine [(4*S*)-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid] and its hydroxyl derivative, hydroxyectoine, and these compounds are of interest as stabilizers for proteins and protectors of membranes from desiccation. The German company bitop AG (www.bitop.de) specializes in making products from osmolytes (e.g., ectoine: Ectoin), and this product is produced on a large scale from *Halomonas elongata* using a technique called bacterial milking (Sauer and Galinski, 1998). The production of ectoine is not limited to halophiles, as it occurs in some cheeses due to growth of *Brevibacterium linens*, which is used in surface ripening of red-smear cheeses. It can accumulate in some cheeses at up to 89 mg per 100 g of cheese (Klein et al., 2007). Other foods may contain ectoine, particularly those in which food is fermented with ectoine-producing microbes under

high-salt conditions, such as in the production of the soy sauce Natto (a Japanese soy product), fermented fish sauces, and cured meat. Ectoine may play a role in preserving these foods, and it may also be used to stabilize food components and improve the freshness of foods (Lentzen and Schwarz, 2006).

8.3. ALKALIPHILES, ACIDOPHILES, AND PIEZOPHILES

The applications of alkaliphilic microorganisms and their bioproducts have recently been reviewed by Sarethi et al. (2011). The best-known application of enzymes from alkaliphiles is that of detergent additives, as they are stable at high pH. True alkaliphiles grow at pH values over 9.0 and have optimal pH values for growth of about 10.0; such organisms have been isolated from environments such as alkaline salt lakes and soils. Some are thermoalkaliphiles, growing at temperatures over 50°C, and others are haloalkaliphilic, growing in high salt. One application relevant to the food industry is the use of alkaliphilic cyclomaltodextrin glucanotransferases (CGTases), which synthesize cyclodextrins (CDs). These molecules are made of rings of α -(1,4)-linked glucopyranose units, with a cavity that facilitates the insertion of various compounds of different sizes and polarities, with different industrial applications in pharmaceuticals and food production. CGTases from the alkaliphile *Bacillus* sp. strain 38-2 have been used to make cyclodextrins (Kato and Horikoshi, 1984) which can be added to many different foods (Szente and Szejtli, 2004), and many other alkaliphilic species have been investigated as sources of CGTases (Salva et al., 1997; Takada et al., 2003).

Acidophiles grow under conditions of low pH, often present in water leached from mines. Others are acidothermophiles, which also require high temperature for growth (e.g., those found in sulfurous hot springs). The archaea *Picrophilus torridus* and *Picrophilus oshimae* grow optimally at pH 0.7 at 60°C and produce heat- and acid-stable glucoamylases (Serour and Antranikian, 2002). *Thermoplasma acidophilum* also produces a thermostable glucoamylase with maximal activity at 75°C (Dock et al., 2008) but difficulties in the large-scale cultivation of thermoacidophilic archaea may ensure that the exploitation of these enzymes may not occur for some time.

Piezophiles are pressure-resistant microorganisms that are found in the deep oceans. However, pressures exceeding 400 MPa are required to denature proteins, and the maximum pressure to which deep-sea microbes are exposed is 120 MPa, so it is unlikely that pressure is a major selection pressure for piezophile proteins (Van den Burg, 2003). It has been suggested that during processing and sterilization of food materials, high pressure of several hundred megapascal may be used to induce the formation of gels or starch granules, denaturation/coagulation of proteins, and transition of lipid phases (Gomes and Steiner, 2004). Flavor and color preservation are also enhanced when high pressure, rather than high temperature, is used in food preparation (Abe and Horikoshi, 2001). Due to difficulties in culturing piezophilic microorganisms, they have few practical applications, so their exploitation in the food industry or for other biotechnological applications awaits new developments in their cultivation.

Radiation is commonly employed to treat food, particularly meats, fruits, and vegetables, to prevent microbial growth, kill pests, and extend shelf life. Foodstuffs are generally irradiated with gamma radiation from a radioisotope source, or bombarded with electrons or x-rays. This has various effects on the foodstuffs, as cellular DNA is damaged by radiation

(Arvanitoyannis et al., 2009). However, one category of extremophile is resistant to this treatment: the radiophiles, or more correctly, radiotolerant organisms (Singh and Gabani, 2011). These organisms contain compounds that may be used in future as radioprotective agents, and furthermore, a thorough knowledge of their mode of protection against damage from ionizing radiation may be applied to ensure that foods are not overirradiated or that appropriate preservation methods are applied to different foods.

8.4. EXTREMOPHILES IN FOOD SPOILAGE AND CONTAMINATION

Food is preserved in various ways, including drying, freezing, refrigeration, fermenting, packaging under modified atmospheres, and curing. The microflora of any foodstuff depends on the preservation method. In particular, the microflora of perishable or preserved food depend on temperature, as many organisms do not grow at the temperatures commonly found in refrigeration and chill cabinets (about 4°C) or in frozen food (approximately –18°C or below). The rate of enzyme-catalyzed reactions is dependent on temperature, and the Q_{10} (temperature coefficient) for enzymatic reactions is generally about 1.5 to 2.5, so that an approximate twofold rise is observed in metabolic rate for every 10°C increase in temperature, until a temperature is reached at which protein denaturation and membrane damage occurs.

In the case of chilled food, psychrotrophic microorganisms tend to predominate over psychrophiles. In food microbiology, it is considered that psychrotrophs are organisms that grow between 0 and 7°C and produce visible colonies or show turbidity over a 7- to 10-day period. Indeed, some of these species can grow at temperatures up to 43°C, which would make them mesophilic. The true psychrophiles are found largely in cold ocean water and in polar regions (Jay et al., 2005). Psychrotrophs can include both food-spoilage organisms and pathogens. The food-spoilage bacteria include a range of bacterial genera, including the gram-negative genus *Pseudomonas*, as well as *Flavobacterium*, *Achromobacter*, *Alcaligenes*, *Escherichia*, and *Aerobacter* spp. (Russell, 1992). Some bacteria can grow, albeit slowly, at freezer temperatures of –12 to –20°C (Michener and Elliott, 1964), and frozen foods containing cryoprotectants may support their growth. The effects of freezing on bacteria in food are complex, and vary with species, with cocci being more resistant than gram-negative rods to freezing (Jay et al., 2005).

8.4.1. Meat and Fish

The growth of psychrotrophic lactobacilli (*Lactobacillus* spp.) and various lactic acid bacteria occurs in packed meat and meat products. These organisms withstand not only relatively low temperatures, but also low pH, the presence of CO₂, nitrite, and salts, and the absence of oxygen (Egan, 1983). Spoilage bacteria, particularly *Pseudomonas*, can give rise to slime on the surface of meat and produce enzymes that convert amino acids to various malodorous compounds. Fungi also contaminate meat: for example, *Cladosporium herbarum* gives rise to black spots on chilled meat (Russell, 1992).

Anaerobic psychrotrophic and psychrophilic *Clostridium* spp. can also give rise to food spoilage and cause food poisoning. *Clostridium estertheticum*, *Clostridium gasigenes*, and *Clostridium algidicarnis* can cause spoilage in vacuum-packed chilled meats, and two psychrophilic species, *C. estertheticum* and *C. gasigenes*, cause “blown pack” spoilage of

vacuum-packed meat (Kalchayanand et al., 1989; Broda et al., 1996, 2000). Blown-pack spoilage occurs when the meat is stored at -1.5 to 4°C for 2 to 4 weeks, causing gas production, pack distention, and a bad smell. The psychrotroph *C. algidicarnis* has been found to affect vacuum-packed meat (Lawson et al., 1994). One PCR-based study indicated that sources of these clostridial species varied from species to species, with some being found on farms and on feces and others being present on environmental samples or chilled dressed carcasses (Broda et al., 2009). Another study showed that 25 different species of psychrotrophs and psychrophiles were present in 431 isolates from Irish beef abattoirs, the commonest being *C. gasigenes*, *C. estertheticum*, and a potentially novel species, TC1 (Moschonas et al., 2011).

Fish in cool temperate waters also carry a largely psychrotrophic and psychrophilic microflora. Some of these are pathogenic, while others can cause spoilage of harvested fish. Psychrotolerant *Pseudomonas* and *Shewanella* spp. are major agents in the spoilage of chilled fish (Gram and Huss, 2000), whereas gram-negative Vibrionaceae are the main culprits in spoilage of unpreserved fish (Gram and Dalgaard, 2002). Salting and addition of benzoate or other preservatives promotes growth of lactobacilli and yeasts (Gram and Dalgaard, 2002). *Aeromonas* spp., psychrotolerant Enterobacteriaceae, *Pseudomonas phosphoreum*, *Shewanella putrefaciens*-like bacteria, and *Vibrio* spp. can all produce trimethylamine-*N*-oxide reductase, which reduces trimethylamine oxide (found in all marine seafood species) to trimethylamine, giving rise to “off” flavors in spoiled fish. These specific spoilage organisms and their spoilage domains can be identified and used to develop methods to predict the shelf life of fish and extend this where possible (Gram and Dalgaard, 2002).

8.4.2. Milk and Dairy Products

Another perishable product that is prone to bacterial contamination is milk. Milk itself is sterile while in the udder (unless pathogenic microorganisms are present) but during milking and storage, milk can become contaminated with bacteria. The diversity of species present depends on the on-farm hygiene practices (Michel et al., 2001). The microflora in milk can be quite diverse and contribute to the organoleptic diversity in raw milk cheeses. In general, the dominant microflora consists of lactic acid bacteria such as *Lactococcus* and *Lactobacillus* spp., as well as *Pseudomonas* spp., *Micrococcus* and *Staphylococcus* spp., and yeasts. The lactobacilli convert the lactose to lactic acid, producing characteristic “sour” milk when milk is allowed to stand at room temperature. However, few of the bacteria that contaminate milk can grow at refrigeration temperatures, including, in general, the lactobacilli. During storage under refrigeration, the psychrotrophs begin to predominate unless the milk has been pasteurized or subjected to ultrahigh-temperature treatment (e.g., 143°C for 3 s). However, this does not fully eliminate bacterial spores (Champagne et al., 1993), and of these, spore-forming *Bacillus* spp. predominate. One study of raw milk stored at 4°C showed that the predominant psychrotrophic species present on the first day of storage was *Pseudomonas fluorescens* (Thomas and Thomas, 1973), which was later shown to be an important agent in the spoilage of milk (Uraz and Citak, 1998). Lafarge et al. (2004) used denaturing gradient gel electrophoresis and temporal temperature gel electrophoresis to show alterations in bacterial composition over time at 4°C , with emergence of psychrotrophic *Listeria* spp. and *Aeromonas hydrophila* populations over 3 days.

Gram-negative psychrotrophs (e.g., *Pseudomonas* spp.) dominate the microflora of stored milk, and have short generation times and the lowest minimum growth temperatures (about -10°C), which are closer to that observed for psychrophiles (Sørhaug, 1992). *Pseudomonas* spp. produce a variety of lipolytic and proteolytic enzymes that can hydrolyze milk fat and proteins. In particular, *P. fluorescens* produces calcium-dependent metalloproteases that preferentially hydrolyze caseins (Koka and Weimer, 2001). Proteolysis by *Pseudomonas* proteases can cause bitterness in milk, due to formation of peptides that impart this flavor (Gobbetti et al., 1995). Lipase hydrolysis of triglycerides can also lead to rancid flavors associated with by-products of lipid hydrolysis (Stepaniak and Sørhaug, 1995). Some of the enzymes produced can withstand pasteurization and ultrahigh-temperature treatment (Braun et al., 1999).

Thermophilic bacilli contaminate dairy manufacturing plants, in particular plants that process milk powder (Burgess et al., 2010). An aerobic plate count at 55°C is generally used to quantify thermophilic bacilli in milk products, but in fact this method comprises two distinct categories: obligate thermophiles, which grow at approximately 40 to 68°C (e.g., *Anoxybacillus flavithermus* and *Geobacillus* spp.) (Flint et al., 2001; Ronimus et al., 2003), and facultative thermophiles, which can grow at thermophilic or mesophilic temperatures. Thermophilic bacilli are important hygiene indicators, because they can form endospores and biofilms, and they also contribute to spoilage of dairy products. Spores are highly resistant to thermal destruction as well as to mechanical disruption and chemicals (Scheldeman et al., 2006). They also display considerable longevity: A sample of milk powder packaged in 1907 and left in the hut of the Antarctic explorer Ernest Shackleton until it was analyzed in 2002 was found to contain viable spores of thermophilic bacilli, largely *B. licheniformis* (Ronimus et al., 2006). The heat resistance properties of spores vary widely from species to species (Burgess et al., 2010) and their properties are difficult to compare, as different trials have used different conditions and temperatures. The only spores found to survive ultrahigh-temperature treatment (134 to 145°C for 1 to 10 s) were *Geobacillus* spp. (Schwarzenbach and Hill, 1999), and of *Bacillus* spp., only *B. sporothermodurans* was not inactivated at temperatures above 120°C for 5 s (Scheldeman et al., 2006).

Biofilm formation is common in dairy manufacturing plants, leading to contamination of milk and milk products. Thermophilic bacilli form monolayer biofilms on stainless steel that is cleaned regularly (Flint et al., 2001), but multilayered structures may also form where milk flow is variable and bacteria can be trapped in milk. (Burgess et al., 2010). Control of thermophilic bacteria presents a challenge, and current methods include shortening production cycles, with more frequent cleaning, using disinfectants, varying temperatures, and reducing the available surface area for microbial growth.

8.4.3. Canned and Dried Food

Some thermophilic bacteria can spoil canned food. In foods containing little acid, some *Bacillus* spp. (e.g., *B. coagulans* and *B. stearothermophilus*) can produce acids. The obligate thermophile *Clostridium saccharolyticum* can produce H_2 and CO_2 , and *C. nigrificans* can produce H_2S . Decreasing the water activity of foods by desiccation or the addition of solutes is a well-established technique for food preservation. However, xerophiles, microorganisms that can grow at low water activity (a_w) levels, which Pitt (1975) defines to be below 0.85 , can colonize dried foods. These comprise yeasts and molds that balance the outside environment with internal solutes, maintaining sufficient osmotic pressure to allow them to

grow. The most xerophilic fungal food-spoilage agent known is *Xeromyces bisporus*, which is capable of growth at an a_w value of 0.61 to 0.62 (Pitt and Christian, 1968). It was first found in liquorice (Fraser, 1953) and has since been discovered in gelatine confectionery, dried fruit, and fruit cake, as well as bakery goods and animal feed (Leong et al., 2011). Any food with a low a_w value (e.g., dried fish, dried fruit, confectionery) that shows growth of a white mold is likely to be colonized by an extreme xerophile. Such fungi are sensitive to high a_w values and cannot be isolated by standard culture methods.

8.5. EXTREMOPHILES AS PATHOGENS OF FOOD SPECIES

Fish that are farmed in cold waters (e.g., those in the North Atlantic) are affected by pathogenic psychrophiles and psychrotrophs. “Winter ulcer” is a disease that affects farmed Atlantic salmon (*Salmo salar*) and farmed cod (*Gadus morhua*). It is caused by *Moritella viscosa* (formerly known as *Vibrio viscosus*). Growth occurs at temperatures from 4 to 21°C, and it requires NaCl for growth (Benediktsdottir et al., 2000). Winter ulcer has been found in Norway, Scotland, Iceland, Canada, and Ireland (Bjornsdottir et al., 2011). The disease occurs primarily in the winter months when water temperatures fall below 7 or 8°C, and it affects juvenile and adult farmed fish. Symptoms include skin ulcers on the scaly part of the fish and around the eyes, as well as hemorrhages, tissue necrosis, and ascites, and it leads to significant morbidity and economic loss (Lunder et al., 1995). To combat this, development of a vaccine has been under way since the 1990s. Oil-based vaccines have been used, but these lead to the development of granulomatous abdominal lesions, which have recently been shown to be associated with an upregulation of innate immunity-related genes and a Th17 immune response (Mutoloki et al., 2010). A study of Icelandic and Norwegian *V. wodanis* and *M. viscosa* strains showed that this organism was a moderate halophile (all strains studied grew at 2 to 4% NaCl) which grows at 4 to 25°C but not at 30°C (Urbanczyk, 2007), which makes it psychrotrophic rather than psychrophilic.

Flavobacterium psychrophilum causes bacterial cold-water disease, found originally in young salmonid fish (e.g., rainbow trout, *Oncorhynchus mykiss*, and coho salmon, *Oncorhynchus kisutch*) in the northwestern United States and Canada in winter and spring, but is known to cause disease on fish farms around the world. An examination of seven strains showed that they all grew between 10 and 20°C, but not at 3°C or above 25°C, making the microorganism a psychrotroph (Bernardet and Kerouault, 1989). It is a gram-negative bacterium with gliding motility, originally called *Cytophaga psychrophila* because of its biochemical characteristics, but was renamed *Flavobacterium psychrophilum* (Bernardet et al., 1996). No commercial vaccines are available, so oral antibiotic therapy is the mainstay of control. However, this may promote the spread of antibiotic resistance in this species. A recent study has shown that under certain conditions it forms biofilms, and that the microorganism is less susceptible to common antimicrobial agents (e.g., oxytetracycline and flumequine) if it is present in a biofilm (Sundell and Wiklund, 2011).

Aliivibrio salmonicida causes cold-water vibriosis, or Hitra disease, particularly in Atlantic salmon. In common with *M. viscosa*, this bacterial species gives rise to outbreaks in winter when the water temperatures drop below 5°C, and continues until temperatures reach 2°C. Fish can show signs of disease at up to 8°C (Noga, 1996). *A. salmonicida* is a facultatively anaerobic motile rod which gives rise to gill pallor, anemia, and hemorrhages, particularly in the integument surround the internal organs, with generalized septicemia

(Egidius et al., 1986). The introduction of an oil-adjuvanted vaccine for cold water vibriosis and furunculosis in Norway in 1994 has largely eliminated outbreaks of this disease.

An extremophile extract may also have applications in the development of an aquaculture vaccine. *Geldibacter algens*, a gram-negative bacterial species found in polar ice, was used in the preservation of a vaccine against *Flavobacterium columnare*, the causative agent of columnaris disease in salmonid fish. *F. columnare* does not make resting spores and is highly sensitive to freezing, making it difficult to lyophilize and ship. The *G. algens* extract was combined with trehalose to make a cryoprotectant that improved the efficiency of lyophilization (Powell et al., 2009).

8.6. CONCLUSIONS

While products from extremophiles, in particular thermophiles and psychrophiles, are beginning to make an impact on the food industry, their true potential remains largely unknown and unexploited. Their economic effect as agents of food spoilage and as pathogens in marine food species is well recognized, and a better knowledge of their physiology and protective mechanisms will lead to better strategies for their control. In the future, extremophile enzymes and other bioproducts may be used extensively in food production and related industries. Their use is limited only by their availability from natural sources, their thermostability, and their ease of expression in heterologous hosts. Once mass-produced and regarded as safe, extremophile products and the microorganisms themselves may find niches in food production as yet unforeseen.

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EXTREMOPHILES AND THEIR APPLICATION TO BIOFUEL RESEARCH

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9.1. INTRODUCTION

The momentum behind biofuels as a sustainable and realistic alternative to conventional fuels (oil, petroleum, natural gas, and coal) continues to grow, driven by the concerns of the longevity and the impact of fossil-fuel use and national fuel security. In a relatively short period (2006–2008), biofuel production and consumption in EU countries has doubled to metric tons tonnes of oil equivalents of 8165 million and 10,064 thousand, respectively (Barnard et al., 2010). Broadly defined as fuels derived from biomass (Ragauskas et al., 2006), biofuels are products of fermentation and metabolism and conventionally include ethanol, butanol, methane, hydrogen, and long-chain alkyl ester derivatives (biodiesel) (Dellomonaco et al., 2010). In the simplest terms, bioalcohols and biodiesel are used as petroleum additives or replacements, while biogases are used for cooking and heating. *Biomass* can potentially refer to any biological material, such as sugarcane, corn, beets, wheat, sorghum, rapeseed, and lignocellulosic wastes. Sugarcane and corn have been used on an industrial scale for fuel production for quite some time (first-generation bioprocessing)

Extremophiles: Sustainable Resources and Biotechnological Implications, First Edition. Edited by Om V. Singh.
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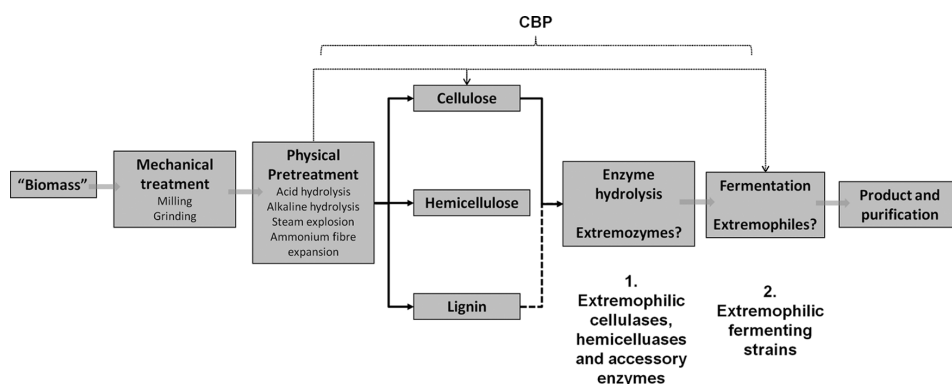


Figure 9.1. Biomass to product: an overview of a typical process. Cellulosic and, to a lesser degree, hemicellulosic fractions of pretreated biomass are targeted for hydrolysis. The fermentable carbohydrate stream is converted to a useful product or biofuel.

utilizing conventional brewing technologies and *Saccharomyces cerevisiae* (Taylor et al., 2009). Despite growing concerns for its reliance on food-grade carbohydrates (Nordhoff, 2007; Tenenbaum, 2008), first-generation processing is anticipated to continue to assist delivery of biofuel targets in the short to midterm future (Ragauskas et al., 2006). New technologies seek to develop biofuels derived from recalcitrant lignocellulosic materials (second-generation processing).

The bioethanol process, from lignocellulosic raw material to product, is depicted in a simple flow diagram in Figure 9.1. Processing encompasses mechanical (Hendriks and Zeeman, 2009; Alvira et al., 2010) physiochemical (Kim et al., 2009; Sierra et al., 2009; Alvira et al., 2010) and enzymological steps (Sun and Cheng, 2002), yielding a wide range of carbohydrates (principally monomeric and polymeric D-xylose, L-arabinose, and D-glucose) for conversion to biofuel through fermentation. Current challenges lie in delivering a fermentable stream of carbohydrate (pentoses, hexoses, and oligomeric sugars) to a catabolically versatile organism with the ability to produce biofuel at high yields (Taylor et al., 2009). Such versatility is innate or may be engineered (Dien et al., 2003; Lee et al., 2008), and coevolution of fermentation inhibitors (such as furfural and hydroxymethylfurfural) is a major concern. Certain aspects of the pretreatment process may be bypassed using a consolidated bioprocess in which one microbial community is employed for both the enzymatic and fermentation steps (van Zyl et al., 2007; la Grange et al., 2010).

The application of extremophiles and extremozymes to the field of biofuel synthesis has long been acknowledged and has recently gained new momentum. Extremophiles thrive in extreme environmental conditions, including low/high pH, low/high temperature, high salinity, and high pressure (Gerday and Glansdorff, 2007). These organisms are often metabolically and physiologically diverse as a function of their adaptive strategies. Extremozymes display kinetic properties that enable integration into processes performed under such extreme conditions as high temperatures, pressures, or solvent concentrations (Demirjian et al., 2001; Egorova and Antranikian, 2005).

In this chapter we focus on the contribution of extremophiles to biofuel production and highlight numerous bacterial strains emerging in industrial circles, principally thermophiles

capable of producing ethanol, butanol, methane, and hydrogen (Ben-Bassat et al., 1981; Taylor et al., 2009; Barnard et al., 2010). Developments in whole-cell biocatalysts are explored, and enzyme diversity, with emphasis on advances made in the last five years, is discussed. We consider future implications and critically evaluate the contribution of extremophiles to this dynamic field of science.

9.2. EXTREMOPHILES AND FUELS

The impact made in biofuel research by extremophiles is dominated by the thermophiles and thermophilic enzymes, with smaller contributions made by psychrophiles and acidophiles. Thermophiles are subclassified as thermotolerant, moderate, or hyperthermophiles. Moderate thermophiles include members of the genera *Geobacillus*, *Thermoanaerobacter*, and *Clostridium* (Rothschild and Mancinelli, 2001; Wagner et al., 2008). Hyperthermophiles are dominated by archaeal species of the genera *Sulfolobus*, *Pyrodictium*, *Pyrolobus*, *Pyrobaculum*, *Thermoproteus*, *Thermococcus*, *Pyrococcus*, and many more (Stetter, 2006a,b). Thermophily reflects innate abilities to adapt, stabilize, and maintain cellular function at high temperature. Tolerance is generally a reflection of protein stability (Ladenstein and Antranikian, 1998), high turnover of DNA, mRNA, and protein, and a host of other molecular adaptations (Madigan and Oren, 1999). Thermophilic strains capable of producing ethanol (Taylor et al., 2009), butanol (Papoutsakis, 2008; Barnard et al., 2010), methane (Weimer and Zeikus, 1977) and hydrogen (Jenney and Adams, 2008) have been identified. Enzymes produced by thermophiles have contributed significantly to developments in bioethanol production. Starch and lignocellulose hydrolysis processes have benefited mostly from enzymes isolated from hyperthermophilic archaea. Psychrophilic enzymes have been applied in starch liquefaction and biodiesel production, while acidophilic enzymes have been used in cellulose deconstruction (Feller and Gerday, 2003; Siddiqui and Cavicchioli, 2006; Cavicchioli, 2011).

9.2.1. Thermophiles and Liquid Fuels: Butanol and Ethanol

When involved in the production of liquid biofuels, thermophiles face the same challenges as mesophilic organisms on a metabolic level. Desirable characteristics include broad catabolic capacity, high alcohol productivity, process hardiness, and alcohol tolerance. Conducting fermentations at high temperatures (50 to 70°C) imparts some advantages; sugars are more readily solubilized and ethanol can be distilled directly, especially through the application of a gas stream or mild vacuum (Hartley and Payton, 1983; Taylor et al., 2009). The presence of sugar- and nutrient-rich feedstocks presents a problem in conventional and ambient fermentations, the best documented being lactic acid bacterial contamination of yeast fermentations (Skinner and Leathers, 2004; Schell et al., 2007). High temperatures reduce gas solubility and favor fermentative metabolism (Banat et al., 1998). Thermophilic ethanologensis is challenged by the availability of strains metabolically engineered for high productivity, product tolerance, and other desirable phenotypes (Taylor et al., 2011). Strains engineered for efficient butanol production under thermophilic conditions are lacking, apparently due to endogenous butanol synthetic pathways being the preserve of mesophilic *Clostridium* spp. (Durre, 2008).

The Genus *Geobacillus*. The genus *Geobacillus* was proposed in 2001 (Nazina et al., 2001), and since then species have undergone several reclassification events, as would be expected in the phylogenetic sorting of a new genus. Fully annotated genomes of *G. kaustophilus* (Takami et al., 2004) and *G. thermodenitrificans* (Feng et al., 2007) have been published. Partially annotated genomes of *G. stearothermophilus* (<http://www.genome.ou.edu/bstearo.html>) and various other *Geobacillus* isolates are publicly accessible via genome banks such as KEGG and NCBI. Members of this genus grow optimally at moderate thermophilic temperatures (55 to 75°C) and are unable to grow at 37°C, fitting the definition of true thermophiles. *Geobacillus* is characterized by a diverse catabolic nature and the ability to ferment and respire anaerobically (McMullan et al., 2004). The excretion of ethanol, albeit at low yield, as part of a mixed fermentative phenotype has often been reported (Cripps et al., 2009; Tang et al., 2009; Taylor et al., 2009). Although an ethanol tolerance of between 3 and 5% v/v is typical of this genus, unique strains with a reportedly high ethanol tolerance, notably *G. thermoglucosidasius* strain MEXG10 (exogenous tolerance to 10% v/v ethanol), have been used in fundamental studies seeking to understand metabolic flux during aerobic, low-aeration, and anaerobic growth (Fong et al., 2006). Identification of key switching points between the Krebs cycle and fermentation have been useful in exploring the metabolism and regulatory mechanisms in *Geobacillus* (Tang et al., 2009). General fermentative metabolic pathways are summarized in Figure 9.2A and B. The fermentative production of organic acids such as lactate regenerates NAD⁺ for glycolysis. In the absence of a functional TCA cycle, the ATP yield from substrate-level phosphorylation is enhanced via the activity of acetate kinase (Tang et al., 2009; Taylor et al., 2009).

The fermentation product profile of *G. stearothermophilus* NCA1503 is comprised largely of lactate, formate, acetate, and ethanol (Hartley and Payton, 1983; Hartley and Sharma, 1987). Early strain development sought the isolation of lactate dehydrogenase (*ldh*)-deficient variants through classical mutagenesis and selection methods (San Martin et al., 1992, 1994). Such an approach proved unstable, in that cultures were reported to revert back to the original phenotype after multiple generations in the absence of the mutagen, fluoropyruvate (San Martin et al., 1994). Stable deletion mutants were, however, developed via targeted molecular disruption of the *ldh* gene (San Martin et al., 1992, 1994).

Significant improvements in the ethanologenic properties of *G. thermoglucosidasius* strains have been achieved. Strategies involved the metabolic engineering of endogenous pathways to improve ethanol yield (Cripps et al., 2009) and the de novo expression of pyruvate decarboxylases (*pdc*) from mesophilic gram-negative species such as *Zymomonas mobilis* (Taylor et al., 2008; Thompson et al., 2008). The latter strategy has been used with great success in *Escherichia coli* and other enterobacteria (Ohta et al., 1991a,b). In *G. thermoglucosidasius*, however, although the *Z. mobilis* *pdc* was expressed successfully, protein misfolding hindered *pdc* expression at temperatures close to the T_{opt} (66°C) value of the strain (Thompson et al., 2008). More recently, studies have focused on the development of genetic methods for the evolution of ethanol-producing variants (Cripps et al., 2009) led by an industrial company, TMO Renewables Ltd. (<http://www.tmo-group.com>). The substitution of the *ldh* promoter with the *pdh* promoter in a *G. thermoglucosidasius* NCIMB 11955 double mutant (Δldh , Δpfl) effectively upregulated *pdh* expression under fermentative conditions (Cripps et al., 2009). Ethanol yields were approaching the theoretical maximum on C₅ and C₆ sugars.

The Genera *Thermoanaerobacterium* and *Thermoanaerobacter*. Fermentative metabolism of *Thermoanaerobacter* and *Thermoanaerobacterium* spp. (Fig. 9.2C and D) generally involves the Embden–Meyerhof pathway and is characterized by the production of mixed acid, alcohol, and hydrogen (Shaw et al., 2008a,b). Strain development for improved ethanol production has focused on *Thermoanaerobacter ethanolicus* (Lacis and Lawford, 1991; Sommer et al., 2004), *Thermoanaerobacter mathranii* (Yao and Mikkelsen, 2010), and *Thermoanaerobacterium saccharolyticum* (Shaw et al., 2008b,2010).

T. ethanolicus ferments both D-glucose and D-xylose (Mai and Wiegel, 2000) and is used as a model organism to understand the relationship between ethanol production, ethanol tolerance, and the role of alcohol dehydrogenase (Burdette et al., 2002; Peng et al., 2008). A primary alcohol dehydrogenase (oxidation of primary alcohols; *adhA*) appears to function primarily during ethanol consumption, while a secondary alcohol dehydrogenase (oxidation of secondary alcohols; *adhB*) functions in ethanol production. A $\Delta adhA$ strain was reported to display high alcohol tolerance (8% v/v) compared to the wild type (<2% v/v), apparently due to a loss of ethanol consumption and an acquired ability to accumulate C₃₀ fatty acids in the membrane (Burdette et al., 1997,2002).

T. mathranii has been reported to grow at up to 70°C and tolerate 4.7% v/v ethanol (Larsen et al., 1997). A Δldh mutant with engineered glycerol dehydrogenase activity (*T. mathranii* BG1L1) has been investigated by a Danish company (<http://www.biogasol.com>) specializing in lignocellulosic-derived products. In addition to the metabolic engineering, mutants demonstrated increased ethanol productivity through long-term adaptation during continuous growth (Klinke et al., 2001; Yao and Mikkelsen, 2010). A substantial quotient of the xylose fraction of lignocellulosic hydrolyzates (corn stover, wheat straw hydrolysate) was shown to be readily converted to ethanol with yields as high as 0.42 g/g (ethanol/sugar).

Significant progress has been made in diverting carbon flow from lactate to ethanol in a *T. saccharolyticum* Δldh mutant strain (Desai et al., 2004). Although the deletion was stable and no lactic acid was produced, ethanol yield was only fractionally higher than in the wild type. Subsequent strain development via disruption of the acetate kinase (*ak*) gene resulted in a homoethanologenic phenotype. These mutants were stable over greater than 150 generations in continuous culture and demonstrated coutilization of D-xylose, D-glucose, mannose, L-arabinose, and ethanol titers of up to 37 g/L (Shaw et al., 2008b). These strains are the intellectual property of Mascoma (<http://www.mascoma.com>).

Thermophilic Species of the Genus *Clostridium*. The development of industrially viable ethanologenic *Clostridium* species suffers from (1) their inability to ferment pentose sugars, and (2) growth inhibition by low amounts of exogenous ethanol (Herrero and Gomez, 1980; Lovitt et al., 1984; Sommer et al., 2004; Demain et al., 2005). Early references to ethanol-tolerant variants of *Clostridium thermocellum* (strain C9) reported a significant improvement in tolerance compared to the parent (2.5% v/v compared to only 0.5% v/v). This phenotype has been linked to increases in normal and ante-isobranched fatty acids in the cell membrane (Herrero and Gomez, 1980; Herrero et al., 1982).

Metabolic pathways associated with *Clostridium* are similar to those of *Thermoanaerobacter* and *Thermoanaerobacterium* spp. Typical fermentative end products include lactate, acetate, ethanol, and under specific growth conditions, hydrogen (Nandi and Sengupta, 1998; Demain et al., 2005). *C. thermocellum* Δldh strains have been reported to be capable of producing approximately 12.5 g/L ethanol at pH 6.5 compared with 5 g/L produced by the parent (Tailliez et al., 1989). Ethanol tolerance was increased from less than 2% v/v

to up to 4% v/v. Concerns with respect to the stability of the *ldh* mutations spurred the development of new genetic tools for strain improvement. The successful electrotransformation (10^5 transformants per microgram of plasmid DNA) of *C. thermocellum* (Tyurin et al., 2004) bodes well for future molecular-based engineering approaches (Tyurin et al., 2006).

The potential use of thermophilic *Clostridia* as high-yielding ethanol producers has been cited widely (Felix and Ljungdahl, 1993; Sommer et al., 2004; Demain et al., 2005). Many are capable of fermentation as well as anaerobic growth in the temperature range 60 to 65°C. Numerous strains are able to degrade cellulose via endo- β -glucanases, exoglucanases, cellobiose phosphorylases, cellodextrin phosphorylases, and β -glucosidases and subsequently ferment the hydrolysis products (Demain et al., 2005). These enzymes may be associated with a multienzyme cellulose-degrading complex or *cellulosome* anchored to the external surface of the cell membrane (discussed in Section 9.3.1). The cellulosome imparts the direct conversion of crystalline cellulose to ethanol and is of great value as a stratagem to achieve a consolidated bioprocess (Felix and Ljungdahl, 1993). The cellulosome of *C. thermocellum* has been characterized extensively (Demain et al., 2005; Blumer-Schuette et al., 2008), and broad catabolic ability has been reported for several strains such as *C. thermocellum* ATCC 27405 and *C. thermohydrosulfuricum* (Herrero and Gomez, 1980; Sato et al., 1992).

9.2.2. Thermophiles and Gaseous Fuels: Methane and Hydrogen

Gaseous fuels have great commercial value (Bagi et al., 2007), and numerous extremophiles with the ability to produce methane and hydrogen have been identified (Chistoserdova et al., 2005; Jenney and Adams, 2008). Methane, also known as biogas, is easily produced on a small scale and is used for electrical, heating, and transportation power. Although the biological production of gas is more commonly cited as a result of microbial consortia, several thermophilic methanogens have been used individually in small-scale digestion trials. These include members of the genera *Methanosarcina* (Ferry, 1997; Lessner et al., 2006; Li et al., 2006; Rohlin and Gunsalus 2010), *Methanococcus* (Kim and Whitman, 1999; Sparling and Daniels, 1986; Miller et al., 1988; Whitman et al., 1997; Yang et al., 2002), and *Methanothermococcus* (Takai et al., 2002). A number of species have been identified as potential candidates for strain development, including *Methanosarcina thermophila* (Ferry, 1997), *Methanococcus jannaschii* (Miller et al., 1988), *Methanococcus thermolithotrophicus* (Sparling and Daniels, 1986), and *Methanothermococcus okinawensis* (Takai et al., 2002). Strain selection for genetic manipulation is reliant on a deep understanding of methane-associated biochemistry and physiology. Biological methane is derived either from the methyl group of acetate with a release of CO₂ (Ferry, 1997; Shima and Thauer, 2005) or from H₂-dependent CO₂ reduction (Chistoserdova et al., 2005).

Biological production of hydrogen by industry involves (1) the biophotolysis of water with thermotolerant algae and cyanobacteria, (2) photosynthetic bacteria for the photofermentation of organic substances, and (3) “dark” fermentation mediated by anaerobic organisms, as shown in Figure 9.3 (Booth, 2005; Carlozzi et al., 2010; Wang et al., 2011). The latter mechanism supports a theoretical maximum yield of four molecules of H₂ per molecule of glucose, possible only with low H₂ partial pressure (p_{H_2}) (Lee et al., 2011; Nanqi et al., 2011). Continuous hydrogen removal is costly, and yields approaching 2 mol per mole of hexose are typical for mesophiles. Close to maximum yield is possible with

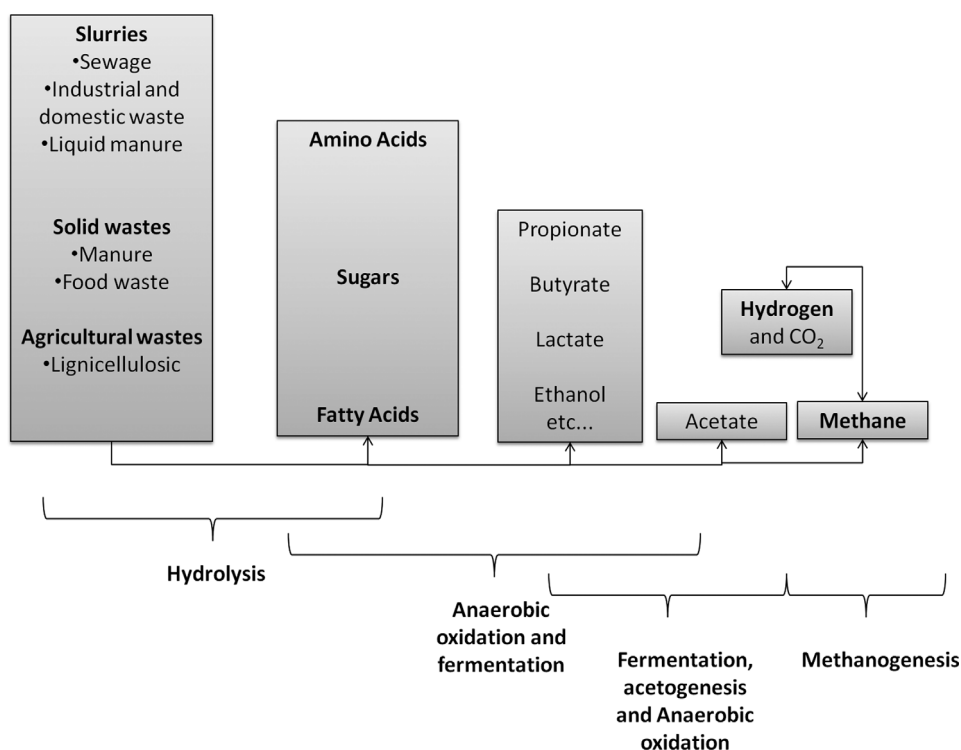


Figure 9.3. The process of “dark fermentation.” Raw slurries and wastes are sequentially converted to amino acids, sugars, and fatty acids and then to organic acids and alcohols. These may be converted to hydrogen and methane by methanogens.

thermophiles, due to reduction in p_{H_2} at high temperature (de Vrije et al., 2007). Variants of *Caldicellulosiruptor saccharolyticus* (Herbel et al., 2010; Willquist et al., 2010) and *Thermotogaelfii* (de Vrije et al., 2009; Schut and Adams, 2009; Nguyen et al., 2010) show particular promise as organisms able to mediate the conversion of organic compounds to hydrogen. Hydrogen capacities of the archaeal genera *Thermococcus* and *Pyrococcus* (Silva et al., 2000; Kanai et al., 2005), and bacterial strains belonging to the genera *Thermoanaerobacter* and *Thermotogales* (Nandi and Sengupta, 1998; Nandi et al., 2001; Bagi et al., 2007; Shaw et al., 2008a) have been reported.

9.2.3. Psychrophiles and Acidophile Whole-Cell Biocatalysts for Fuels

Psychrophiles grow optimally below 10°C, and acidophiles prefer conditions below pH 2 (Siddiqui and Cavicchioli, 2006). Considering that much of the globe is ocean covered (averaging 2 to 5°C) and a fifth of the land surface is polar (Feller and Gerday, 2003), psychrophiles represent a large resource of potentially novel genes and enzymes. The physiology, isolation, biodiversity, and adaptive qualities of psychrophiles (Cavicchioli et al., 2000, 2006; Cavicchioli, 2006) and acidophiles (Matin, 1999; Golyshina et al., 2006; Kim and Peeples, 2006; Baker-Austin and Dopson, 2007) have been reviewed elsewhere. While

these organisms are exploited for their specialist enzymes in the biotechnology sector (Cavicchioli et al., 2010), applications in the biofuel industry are limited. A “cold” biofuel process may be attractive when low-temperature starch liquefaction or *cold hydrolysis* is involved (Galvez, 2005; Williams, 2006). Genencor developed the Stargen line of cold-active fungal enzymes, which includes a range of amylases and glucoamylases that hydrolyze starch in a simultaneous saccharification and fermentation process (Williams, 2006; Huston, 2008). Application of enzyme producers as cellular biocatalysts is hindered by the lack of genetic tools, methods, and markers required for molecular strain development. Exceptions include the use of the acidophile *Acidiphilium cryptum* as a microbial fuel cell for the production of electricity, using iron reduction as an anode biocatalyst (Borole et al., 2008).

9.3. EXPLOITING EXTREMOPHILIC ENZYMES IN BIOMASS CONVERSION TO BIOFUEL

The selection of appropriate feedstocks is critical to the design of cost-effective biofuel processes. Based on their chemical structure, each feedstock has different characteristics which need to be identified in order to maximize their conversion efficiencies to biofuels. The application of extremophiles, particularly their robust enzymes, has enormous potential in addressing the challenges associated with the biochemical conversion of biomass, critical for successful commercial development. Outlined below is the recent literature on the development and use of extremophilic enzymes for biofuel production, most of which are involved in depolymerization of the various feedstocks.

9.3.1. Lignocellulose: A Recalcitrant but Valuable Biomass Resource

First-generation biofuels are sourced from corn grain (starch) and sugarcane (sucrose). The sensitivity over the use of food grains for fuel has stimulated interest in second-generation fuels derived from abundant lignocellulosic materials such as agricultural, forest, and urban wastes (Haber, 2007; Nordhoff, 2007; Tenenbaum, 2008). Plant cell walls are the source of lignocellulose, which is comprised of cellulose (35 to 50% of plant dry weight), hemicellulose (20 to 35%), and lignin (5 to 30%) (Perez et al., 2002; Lynd et al., 2005). Second-generation biofuel production is gaining huge momentum and is recognized as a “green,” secure, and sustainable fuel alternative (Farrell et al., 2006; Hill et al., 2006). The main bottleneck in a second-generation process is efficient conversion of recalcitrant lignocellulosic biomass into fermentable sugars (Garcia-Aparicio et al., 2007; Merino and Cherry, 2007). This may be achieved by combining pretreatment strategies: for example, steam explosion or dilute acid exposure, with enzyme hydrolysis and downstream fermentation (Hendriks and Zeeman, 2009; Yang et al., 2009; Alvira et al., 2010). Physical, chemical, physicochemical, and biological processes have been used for the pretreatment of lignocellulosic materials, a process that may employ extreme conditions of temperature, pH, pressure, or biotoxic chemicals (Eggeman and Elander, 2005; Hendriks and Zeeman, 2009). Pretreatment partially solubilizes hemicellulose and cellulose fractions, while removing lignin components (Mosier et al., 2005a,b). When coupled to effective enzyme hydrolysis, harsh pretreatment conditions may be reduced (Garcia-Aparicio et al., 2007; Merino and Cherry, 2007). Harsh process conditions have been a driving force behind the development

of extremophilic whole-cell biocatalysts and the industrial use of extremozymes, the latter being discussed in the remainder of the chapter. Industry requires enzymes with high specific activity, low sensitivity to end-product inhibition, and reasonable stability and flexibility when placed in fluctuating process conditions (Turner et al., 2007; Kuhad et al., 2011; Parawira and Tekere, 2011). Enzymes isolated from thermophilic and acidophilic sources are gaining interest due to “up-front” delivery of such desirable characteristics (Lynd et al., 2008). For in-depth information the reader is referred to excellent and recent reviews (Gomes and Steiner, 2004; Antranikian et al., 2005, 2009; Lynd et al., 2005; Antranikian and Egorova, 2007; Wilson, 2009; Yeoman et al., 2010).

A wide range of glycosylhydrolases (GHs), including cellulases and hemicellulases, are involved in the deconstruction of lignocellulose to monosaccharides (D-xylose, L-arabinose, and D-glucose). GHs are largely characterized by similarity in catalytic domains and carbohydrate-binding modules (Davies et al., 2005). Based on the arrangement and positioning of active sites, GHs can be separated into three catalytic domain architecture types: (1) tunnel, specialized for processive hydrolysis where reducing and nonreducing ends of the substrate are fed into the active site of the enzyme; (2) cleft, specialized for nonprocessive internal hydrolysis; and (3) crater or pocket for end-on-attack hydrolysis, releasing monosaccharides (Davies et al., 2005).

Cellulose Deconstruction. Cellulose is a glucan homopolysaccharide comprised of β -D-glucopyranose units linked together by β -(1,4)-glycosidic bonds (Kuhad et al., 1997). Cellulases hydrolyze glycosidic bonds of amorphous and crystalline cellulose (Jones, 1964; Mischnick and Momcilovic, 2010), and depending on the type of cellulase, different regions and cellulose chains are targeted. Cleft-shaped catalytic domains of endoglucanases [(1,4)- β -D-glucan 4-glucanhydrolases, EC 3.2.1.4] hydrolyze internal glycosidic bonds within soluble amorphous cellulose, releasing cellulooligosaccharides. Exoglucanases [(1,4)- β -D-glucan cellobiohydrolase, EC 3.2.1.91] display tunnel-shaped catalytic modules that progressively release cellobiose residues from the reducing and nonreducing ends of cellulose chains (Divne et al., 1993, 1994, 1998; MacKenzie et al., 1998; Teeri et al., 1998). A few exoglucanases have been shown to open tunnel-shaped catalytic sites (Davies et al., 1995), while “cleft-shaped” endoglucanases have been identified that are able to degrade cellulose end residues processively (Hakamada et al., 2002; Gilad et al., 2003). Cellulases may therefore be grouped according to preferred substrates, with exoglucanases being more specific for crystalline cellulose and endoglucanases for soluble amorphous cellulose. Exoglucanase and endoglucanase endproducts (cellobiose and cellodextrins) are either hydrolyzed extracellularly by β -glucosidases (EC 3.2.1.21), or transported across the cell membrane and metabolized intracellularly (Lee et al., 1988; Woodward et al., 1990).

Thermostable endoglucanases have been described for various archaeal and bacterial genera, including *Pyrococcus* (Ilari et al., 2009; Kim and Ishikawa, 2010), *Sulfolobus* (Huang et al., 2005), *Thermotoga* (Chhabra and Kelly, 2002; Chhabra et al., 2002), *Geobacillus* (Rastogi et al., 2011), and *Thermus* (Antranikian and Egorova, 2007). Numerous thermophilic exocellulases and glucosidases have been reported, including examples from *Pyrococcus* and *Thermus* spp. (Costantino et al., 1990; Chang et al., 2001; Xiangyuan et al., 2001; Hong et al., 2007). Protein engineering has yielded enzymes with significantly improved specificity and activity (Table 9.1). Large quantities of free enzymes are required to synergistically degrade complex substrate such as lignocelluloses (Lynd et al., 2002).

TABLE 9.1. Engineered Cellulases and Xylanases Using Rational Design and Directed Evolution

Source of Enzymes	Enzyme Function	Type of Modification	Relative Modification of Activity/Stability	Substrate ^a	References
<i>Rational Design</i>					
<i>Acidothermus cellulolyticus</i>	Cel5A endoglucanase	Site-directed mutation that reduced the binding efficiency of product for the enzyme	Reduced cellobiose product inhibition and increased hydrolysis by 40% through synergism with <i>T. reesei</i> exoglucanase I	CMC and PASC	Baker et al., 2005
<i>Paenibacillus polymyxa</i>	Multi-functional Cel44C–Man26A	Truncation from 1352 to 549 amino acids	Reduced the size of the enzyme, simplified recombinant expression	CMC, OSX, LBG, and LIC	Cho et al., 2008
<i>Pectobacterium chrysanthemi</i>	Cel5Z endoglucanase	Truncation removing the CBM 426 to 280 amino acids	Increased activity by 80% on CMC while reduced activity on Avicel	CMC	Lim et al., 2005
<i>Thermobacillus xylanolyticus</i>	β -Xylanase	Introduction of disulfide bridges	Increased thermostability half-life to 70°C for 180 min, 10-fold higher than the wild type with almost twice the specific activity	BW xylan	Paes and O'Donohue, 2006
<i>Thermobifida fusca</i>	Cel6B exoglucanase	Double site-directed mutation G234S and G284P	Increased catalytic activity two-fold on PASC and three-fold on FP	PASC and FP	Zhang et al., 2000
	Cel9A endo/exoglucanase	CBM F476Y substitution	Increased the relative activity by 40%	CMC and PASC	Escovar-Kousen et al., 2004
	Cel9A processive endoglucanase	Site-directed mutagenesis, combining catalytic and CBM mutations	An increase of 150% in catalytic activity on BCC and 200% on PASC; increased synergism up to 330% when combined with an endoglucanase	BCC and PASC	Li et al., 2010
<i>Thermotoga maritima</i>	Cel5A endoglucanase	Site-directed mutation that increased enzyme acidity	10% increase in activity; retained full activity until 18 h of incubation at 80°C	CMC	Mahadevan et al., 2008
	Cel5A endoglucanase	Separate additions of CBM1 and CBM6	Increased activity 14 to 18-fold; both retained full activity until 18 h of incubation at 80°C	Avicel	Mahadevan et al., 2008

(continued)

TABLE 9.1. (Continued)

Source of Enzymes	Enzyme Function	Type of Modification	Relative Modification of Activity/Stability	Substrate ^a	References
<i>Bacillus subtilis</i>	XylA xylanase	Directed Evolution epPCR and DNA shuffling	Melting temperature increased 20%	OSX	Ruller et al., 2008
<i>Bacillus subtilis</i> (BSE616)	Endoglucanase		Increased the relative catalytic activities of fused (five-fold) and free enzymes (2.2-fold)	CMC	Kim et al., 2000
<i>Clostridium cellulovorans</i>	Cellulosomeendoglucanase (EngB)		Thermostability of the mutant EngB was increased up to seven-fold	CMC	Murashima et al., 2002a,b)
<i>Clostridium cellulovorans</i> and <i>C. thermocellum</i>	Endoglucanase (EngD) and endoglucanase (EngE)		Increased relative activity by 10 to 20% and thermostability of the enzyme up to 3.1-fold	CMC	Lee et al., 2010a,b
<i>Paenibacillus polymyxa</i>	β -Glucosidases		20-fold increase in thermostability and eightfold increase in activity	pNPG	Arrizubieta and Polaina, 2000
<i>Pyrococcus furiosus</i> <i>Reticulitermes speratus</i> , <i>Nasutitermes</i> <i>takasagoensis</i> , <i>Coptotermes formosanus</i> and <i>C. acinaciformis</i>	CelB β -glucosidase Endoglucanase	Gene shuffling DNA family shuffling of the four genes	Increased activity up to fivefold Increased activity 20 to 30-fold	pNPG CMC	Kaper et al., 2000 Ni et al., 2005
<i>Streptomyces halstedii</i> JMB	Xys 1 xylanase	EpPCR G133D and N148D mutations	Increase of 22 to 25% in specific activity	RBB-xylan	Diaz et al., 2004a,b

^aCMC, carboxymethyl cellulase; pNPG, *p*-Nitrophenol- β -D-glucopyranoside; FP, filter paper; PASC, phosphoric acid swollen cellulose; BCC, bacterial crystalline cellulase; BW xylan, birchwood xylan; RBB-xylan, Remazol Brilliant Blue coupled to birchwood xylan; LBG, locust bean gum; LIC, lichenan.

Therefore, currently, most industrial cellulases are produced by engineered strains of aerobic fungi, specifically *Trichoderma reesei* (syn. *Hypocrea jecorina*) and *Humicola insolens*, due to very high yields and high specific activities (Karlsson et al., 2002).

Hemicellulose Deconstruction. Hemicellulose consists of a combination of complex branched heteropolymers (Beg et al., 2001). Xylan and glucomannan constitute the largest components in hardwood and softwood, respectively. Other components include xyloglucan, galactoglucomannan, and arabinogalactan. Hemicelluloses are hydrolyzed by a combination of enzymes releasing valuable fermentable sugars such as pentoses (D-xylose and D-arabinose) and hexoses (D-glucose, D-galactose, and D-mannose) as well as sugar acids (Subramanian and Prema, 2000, 2002). The xylan β -(1,4)-linked backbone and respective side chains are hydrolyzed by a combination of endoxylanases (EC 3.2.1.8), β -xylosidases (EC 3.2.1.37), α -glucuronidases (EC 3.2.1.139), α -L-arabinofuranosidases (EC 3.2.1.55), and acetylxyLANesterases (EC 3.1.1.72). Glucomannans, in turn, are hydrolyzed by β -mannanase (EC 3.2.1.78) and β -mannosidase (EC 3.2.1.25) (Subramanian and Prema, 2002). The growing importance of xylanolytic enzymes in industry lies in the assistance that xylanases provide in disassociating lignin from the cellulose and hemicellulose fractions. Xylanases are, however, not commonly sourced from extremophiles. A hyperthermophilic *Pyrodictium abyssi* xylanase shows optimal activity at 110°C (Andrade et al., 2001) and thermophilic examples from the genera *Pyrococcus*, *Thermococcus*, and *Sulfolobus* have been reported (Uhl and Daniel, 1999; Bergquist et al., 2001; Cannio et al., 2004; Kambourova et al., 2007; Maurelli et al., 2008). Xylanolytic thermophiles belonging to the genera *Clostridium*, *Rhodothermus*, and *Thermotoga* have also been reported (Karlsson et al., 2004; Liebl et al., 2008; Fan et al., 2009; Okazaki et al., 2010). Despite the apparent paucity of xylanase activities, associated activities (e.g., acetyl xylan esterase and arabinofuranosidase) are often reported (Antranikian et al., 2005).

Lignin Deconstruction. Lignin has a highly recalcitrant complex aromatic structure (Wong, 2009) synthesized by the oxidative coupling of three aromatic alcohol precursors: coniferyl alcohol, sinapyl, and *p*-coumaryl (Bhuiyan et al., 2009). Precursors form, respectively, guaiacyl, syringyl, and hydroxyphenylphenylpropanoid subunits (Martinez et al., 2005). Although of little use as a source of fermentable carbon, lignin needs to be removed for efficient biomass processing, as it effectively binds the cellulose and hemicellulose into complex matrices. Numerous microorganisms have been implicated with the ability to degrade lignin by enzymatic and oxidative nonenzymatic mechanisms. Fungi (e.g., basidiomycetes) may hydrolyze lignin with the help of lignin peroxidases (EC 1.11.1.14), manganese peroxidases (EC 1.11.1.13), versatile peroxidases (EC 1.11.1.16), and phenol oxidases (laccases) (EC 1.10.3.2) (Martinez et al., 2005). Several accessory enzymes, such as cellobiose dehydrogenase (EC 1.1.99.18), glyoxal oxidase (EC 1.2.3.5), aryl alcohol oxidase (EC 1.1.3.7), and cellobiose/quinoneoxidoreductase (EC 1.1.5.1), are involved in oxidative degradation of lignin (Martinez et al., 2005). These enzymes are capable of producing H₂O₂ that releases hydroxyl (\cdot OH) free radicals, which in turn degrade the lignin polymer nonspecifically (Guillen et al., 2000a,b; Gomez-Toribio et al., 2009).

Complex Synergism: The Cellulosome. Bacterial cellulase production processes are commonly found in anaerobic clostridia, including *C. thermocellum*, *C. cellulolyticum*, and *C. cellulovorans* (Shoham et al., 1999). Clostridia are able to degrade natural crystalline

cellulose through complexed synergism, implying that the cellulolytic process as a whole is more efficient than the sum of the individual cellulase enzymes (Boisset et al., 1999; Demain et al., 2005). Synergism derives from cellulolytic machinery complexed within large regulated multienzyme structures called *cellulosomes* (Felix and Ljungdahl, 1993; Boisset et al., 1999; Demain et al., 2005; Blumer-Schuetz et al., 2008; Xu et al., 2010). A cellulosome consists of a multidomain scaffoldin that carries at least one carbohydrate-binding module and several cohesion modules. Functionally different GHs share a highly conserved dockerin module which binds to cohesion modules displayed on the scaffoldin, resulting in a large multifunctional enzyme complex. Multiple cellulosomes are displayed on the cell surface and ensure proximity between synergistic compounds. Catalytic enzymes that bear dockerin modules are able to bind to complementary cohesin modules through highly specific “plug and socket” cohesion–dockerin interactions (Leibovitz and Beguin, 1996; Pages et al., 1997, 1999). Although produced primarily by clostridia (Bayer et al., 1983; Lamed et al., 1983), cellulosomes have been reported for *Acetivibrio cellulolyticus* (Xu et al., 2004) and *Ruminococcus flavefaciens* (Jindou et al., 2006), both of which are able simultaneously to hydrolyze and ferment lignocellulosic-derived sugar residues into ethanol at high temperatures. The use of cellulosomes in lignocellulose degradation has several advantages over free enzyme systems (Lynd et al., 2002). The close proximity of cellulosome enzymes increases synergism by reducing nonproductive adsorption and product inhibition (Shoham et al., 1999). Synergistic interactions between free enzymes, on the other hand, are diluted by large substrate volumes. These complexes have inspired the formulation of designer cellulosomes with enhanced substrate binding, proximity between enzymes, and functional diversity (e.g., structures with cellulolytic and xylanolytic activity) (Maki et al., 2009). Such studies contribute to the development of a consolidated bioprocess aimed at enabling microbes to effectively convert cellulose into bioethanol in a single step (Lynd et al., 2008).

9.3.2. Other Polymeric Biomass Resources

Lignocellulose is currently the major target for deconstruction and subsequent extraction and production of bioproducts. Other polymeric substrates do, however, warrant discussion, in particular those with relevance to biofuel production. These include starch, lipids, and chitin.

Starch Hydrolysis. Starch hydrolysis can result in a variety of products, principally glucose, fructose, dextrins, maltose, and trehalose (Antranikian and Egorova, 2007). Thermophilic enzymes may be applied to act in synergy with a high-temperature starch solubilization process, while psychrophilic enzymes may assist in the cold starch hydrolysis process (discussed in Section 9.2.3). The enzyme α -amylase (EC 3.2.1.1) hydrolyzes starch polymers to a variety of oligomeric chains. Thermophilic α -amylases have been reported primarily for hyperthermophilic archaea of the genera *Methanocaldococcus*, *Pyrococcus*, *Sulfolobus*, and *Thermococcus* (Laderman et al., 1993; Leveque et al., 2000; Yang et al., 2004; Kim and Peeples, 2006; Synowiecki et al., 2006; Van et al., 2007). Optimal activity typically resides around 90°C, and the enzymes display impressive thermostability, with activity being retained after 4 h of autoclaving (120°C). Notable examples include amylases from *Methanocaldococcus jannaschii* (a T_{opt} value of 120°C and a $T_{1/2}$ value of 50 h at 100°C), *Pyrococcus furiosus* (T_{opt} of 100°C and $T_{1/2}$ of 13 h at 98°C) and

Thermococcus kadakaraensis (T_{opt} of 90°C and $T_{1/2}$ of 24 h at 70°C) (Antranikian et al., 2005; Antranikian and Egorova, 2007). A moderately thermostable amylase from *Bacillus licheniformis* warrants mention due to extensive use in industry (Bravo Rodriguez et al., 2006). A number of halophilic enzymes tolerant of high salt and solvent concentrations have been reported (Li et al., 2002; Amoozegar et al., 2003; Tan et al., 2003; Antranikian and Egorova, 2007). Amylases from haloarchaea have been reported to display activity in various solvents (toluene, benzene, and chloroform) and to tolerate up to 4.5 M NaCl and pH values as high as 10 (Antranikian et al., 2005; Antranikian and Egorova, 2007). Glucoamylase (EC 3.2.1.3) hydrolyzes terminal residues on α -(1,4)-linked D-glucose chains, liberating D-glucose. Thermostable and acidophilic glucoamylases from the archaeal genera *Sulfolobus* (Kim et al., 2004), *Picrophilus* (T_{opt} of 90°C and $T_{1/2}$ of 24 h at 90°C and pH_{opt} of 2) and *Thermoplasma* (T_{opt} of 75 to 90°C and $T_{1/2}$ of 24 to 40 h at 60 to 90°C, pH_{opt} of 2 to 5) are particularly noteworthy (Serour and Antranikian, 2002). The enzyme α -glucosidase (EC 3.2.1.20) hydrolyzes α -(1,4)-bonds in dimers, trimers, and tetramers of sugars such as D-glucose and maltose. Members of the genera *Pyrococcus*, *Sulfolobus*, *Ferroplasma*, and *Thermococcus* produce highly thermotolerant examples (Costantino et al., 1990; Kengen et al., 1993; Piller et al., 1996; Galichet and Belarbi, 1999; Schiraldi et al., 2000; Chang et al., 2001; Giuliano et al., 2004; Ferrer et al., 2005; Golyshina et al., 2006). A number of moderately thermophilic variants have also been reported for *Thermoanaerobacter*, *Thermotoga*, and *Clostridium* (Sharmila et al., 1998; Lodge et al., 2003; Thompson et al., 2004; Hong et al., 2007; Zhou et al., 2009).

Transesterification of Oils. The most commonly used method for biodiesel production is the transesterification of oils or fats involving lipases as a catalyst. This approach often produces a highly pure product that is easily separated from the glycerol by-product (Bisen et al., 2010). Lipases (EC 3.1.1.3) differ from esterases (EC 3.1.1.1), primarily in their preference for substrate chain length. Lipases prefer longer-chain “true lipids,” while esterases are more active on short-chain esters. A huge variety of lipases and esterases has been reported, and many are derived from extremophiles (Haki and Rakshit, 2003). Thermophilic examples include enzymes described for *Thermoanaerobacter* spp. (Royter et al., 2009; Rao et al., 2011) and the archaeal genus *Sulfolobus* (Huddleston et al., 1995; Sehgal and Kelly, 2002; Ejima et al., 2004; Mandrich et al., 2007). Commercial use of psychrophilic lipases (Huston, 2008; Cavicchioli et al., 2010) has been moderate save for a few well-known examples such as the *Candida antarctica* lipases available from Sigma Aldrich and Novozymes. Although the main driver has been the need for low-temperature active enzymes in the detergent and textile industries (Huston, 2008), the *C. antarctica* lipases have been used in the production of biodiesel (Akoh et al., 2007; Fjerbaek et al., 2009). The cost of the enzyme, however, remains the major barrier for industrial implementation of biodiesel. Several strategies, including enzyme immobilization and protein engineering, are being explored to develop this as a commercially feasible source of biodiesel.

Chitin. Chitin is one of the most abundant natural polymers and can be found in marine shells, crustacean shells, and insect exoskeletons. As yet, chitin remains a largely untapped resource. Chitin-degrading enzymes such as chitin hydrolase (EC 3.2.1.14), chitin oligomer hydrolase (EC 3.2.1.52), and *N*-acetyl-D glucosaminidase or chitinase (EC 3.2.1.30) act upon β -(1,4)-homopolymers of *N*-acetyl glucosamine. Many archaea grow on chitin and produce the enzymes responsible for chitin breakdown (Imanaka et al., 2001;

Andronopoulou and Vorgias, 2004; Blumer-Schuetz et al., 2008; Nakamura et al., 2008). Multienzyme complexes analogous to the cellulosome have been reported (Andronopoulou and Vorgias, 2004; Blumer-Schuetz et al., 2008; Kikkawa et al., 2008). High-temperature optima, thermostability, solvent tolerance, and broad substrate specificity are characteristics of these enzymes. Bacterial variants have been described for members of the genera *Clostridium* and *Rhodothermus* (Hobel et al., 2005; Taylor et al., 2006; Dvortsov et al., 2009,2010).

Alcohol Dehydrogenases and Monomeric Sugar Isomerases. Alcohol dehydrogenases (ADHs EC 1.1.1.1) have applications in the production of ethanol, butanol, and secondary alcohols. *Thermoanaerobacter* and *Geobacillus* are known to possess several ADHs, the study of which has assisted discrimination of specific activities (i.e., ethanol production and consumption, secondary alcohol production, etc.) (Lamed and Zeikus, 1981; Arni et al., 1996; Burdette et al., 1997,2002; Talarico et al., 2005; Jeon et al., 2008; Peng et al., 2008). Examples from archaea are less common, but notable enzymes include those reported for *Sulfolobus solfataricus* (Ma and Adams, 2001; Ohshima et al., 2001; Raia et al., 2001; Esposito et al., 2002) and *Thermococcus hydrothermalis* (Antoine et al., 1999), most being alkaliphilic and thermostable with high-temperature optima (Giordano et al., 2005; Secundo et al., 2005).

Sugar isomerases play a significant role in tailoring carbohydrate streams (glucose, xylose, or arabinose) to respective monomeric forms (fructose, xylulose, or ribulose). Glucose isomerase is currently used widely in the production of high-fructose corn syrup in the food industry (Bhosale et al., 1996; Lim and Saville, 2007), while arabinose isomerase is used in the production of sweeteners (Kim, 2004). Since high production yields rely on high process temperature ($>100^{\circ}\text{C}$), examples reported for *Thermoanaerobacterium* (Lee et al., 1993; Lloyd et al., 1994; Vieille et al., 1995; Liu et al., 1996) and *Thermotoga* (Vieille et al., 1995; Chayen et al., 1997; Hess et al., 1998) are of particular relevance. Although not widely cited as of use to the fuel industry, their demonstrated ability to selectively alter sugar streams may be of use in harmonizing the majority sugar content within a hydrolysate, with the catabolic state of the fermenting organism.

9.3.3. Enhancing Enzyme Efficiency

Despite the potential advantages offered by extremozymes for application in biofuels, enzymes often require modification to achieve optimal activity in a particular process so as to improve process economics. Engineering of extremophilic enzymes is a routine approach taken by companies such as Novozymes and Verenum (Banerjee, 2010). The properties of individual enzyme components may be improved by rational design or directed evolution. Rational design relies on detailed knowledge of protein structure and structure–function relationships, ideally from high-resolution crystallographic studies (Zhang and Fang, 2006). Even with detailed knowledge of the enzyme structure, targets for rational design are difficult to predict. Directed evolution does not require detailed knowledge of enzyme structure or interactions between enzyme and substrate, but rather, employs selective pressures, such as pH, thermostability, and catalytic activity to “evolve” enzymes according to desired characteristics. Directed evolution relies on the creation of large mutant libraries with DNA mutation techniques such as error-prone PCR (epPCR), DNA shuffling, or the staggered extension process (StEP) PCR (Zhao and Zha, 2006). Examples of enzymes with altered properties are summarized in Table 9.1. Rational and irrational

designs have resulted in cellulases and hemicellulases with increased catalytic activity, enzyme stability, recombinant expression, and tolerance of hydrolysis product inhibition (Maki et al., 2009). Specific modules targeted within enzymes include carbohydrate-binding molecules, catalytic sites, and surface structures (Kittur et al., 2003). The Schema structure-guided recombination uses a modeling approach to generate novel enzymes by randomly shuffling “blocks” of amino acids between structurally closely related parent proteins (Meyer et al., 2006). This approach was designed to reduce the number of inactive clones by limiting the extent of conformational disruption of the tertiary structure. Heinzelman et al. (2009a) applied Schema in the generation of novel class II exoglucanases by combining sequence information from enzymes originating from different species. Shuffling yielded 15 novel enzymes with better catalytic performance and thermostability than those of parents (Heinzelman et al., 2009a,b). In addition, thermostability has been studied by comparing the structures of endoglucanases and lipases from several different sources (Danson and Hough, 1998; Ladenstein and Antranikian, 1998; Crennell et al., 2002; Sinchaikul et al., 2002; Tyndall et al., 2002; Volkers et al., 2009). General trends and potential strategies for increasing internal stabilization have been reviewed and include an increase in ion-pair networks, disulfide and salt bridging, hydrogen bonding, hydrophobic and aromatic interactions, and stabilization of surface-exposed amino acids (Ladenstein and Antranikian, 1998; Li et al., 2005).

9.4. CONCLUSIONS AND FUTURE PROSPECTS

Extremophiles' contributions to biofuel production have become tangible and are growing fast. The development of thermophilic whole-cell biocatalysts of industrial relevance is one of the most significant recent contributions. Developments in genetic systems and strain production will continue to push new innovations to achieve a sustainable supply of biofuels. The wide range of enzymes required for lignocellulose and polymeric deconstruction presents a challenge to biofuel production. The benefits associated with high-temperature hydrolysis, such as energetic advantages, reduced risk of contamination, and high production rates, coupled with the use of enzymes or organisms able to utilize a wide substrate range, suggest that thermophilic ethanologens have the potential to overcome commercial barriers to second-generation biofuels.

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SUSTAINABLE ROLE OF THERMOPHILES IN THE SECOND GENERATION OF ETHANOL PRODUCTION

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10.1. INTRODUCTION

Regular gasoline shortages, price hikes, contemporary industrial developments, the rapid pace of urbanization, and environmental pollution call for environmentally sustainable energy sources (Goldemberg, 2007; Chandel et al., 2010a). Currently, gasoline makes up the greatest fraction of the global energy supply. Looking at the current fossil-fuel reserves/production ratio, oil, natural gas, and coal reserves are expected to be exhausted in nearly 40, 60, and 130 years, respectively (www.bp.com). Ethanol made from biomass, called second-generation ethanol, has environmental, economic, and geopolitical benefits and is considered the safest and cleanest liquid fuel alternative to fossil fuels (Chandel et al., 2010a; Lynd and Woods, 2011). The energy crisis in the 1970s alarmed the major industrial

TABLE 10.1. Top Ten Countries in World for Ethanol Production (Millions of Gallons per Year)^a

Country	Year			
	2007	2008	2009	2010
United States	6,498.60	9,000.00	10,600.00	13,230.00
Brazil	5,019.2	6,472.2	6,577.89	6,921.54
European Union	570.30	733.60	1,039.52	1,176.88
China	486.00	501.90	541.55	541.55
Thailand	79.20	89.80	435.20	NA
Canada	211.30	237.70	290.59	356.63
India	52.80	66.00	91.67	NA
Colombia	74.90	79.30	83.21	NA
Australia	26.40	26.40	56.80	66.04
Others	NA	NA	247.27	NA
World total	13,101.7	17,335.20	19,534.993	22,946.87

Source: Modified from F.O. Lichts; cited in Renewable Fuels Association, 2007–2010.

^aNA, not available.

countries of the world, particularly the United States, and led to the first investigations of sustainable energy solutions.

In need of converting cellulosic feedstock into fuel ethanol, Brazil launched its National Alcohol Program, ProAlcool, to include ethanol derived from sugarcane to reduce the burden of gasoline, which is now quite successful. Today, 44% of the Brazilian energy matrix is renewable, and 13.5% is derived from sugarcane (Goldemberg, 2007; Soccol et al., 2010). Currently, the United States is the world leader in bioethanol production (13 billion gallons, primarily from corn), followed by Brazil (8 billion gallons) (Soccol et al., 2010). The European Union has planned 10% ethanol blending by 2020 (Taylor et al., 2009). Other countries, including China, India, and South Africa, are implementing similar initiatives. Currently, ethanol is being produced from corn in the United States and from sugarcane in Brazil, India, and South Africa (Chandel et al., 2010a; Soccol et al., 2010; Lynd and Woods, 2011). Table 10.1 summarizes worldwide fuel ethanol production for 2007–2010. The increased demand for energy and the food and feed values of the respective agromaterials require research into harnessing by-products such as corn stover, sugarcane bagasse and leaves, or other cellulosic feedstock for ethanol production (Graham-Rowe, 2011). Cellulosic feedstocks have the capability to replace about 30% of petroleum used in the United States (<http://feedstockreview.ornl.gov>).

There is a copious amount of cellulosic biomass worldwide that can be exploited for fuel ethanol production. The steps required for this process include pretreatment of biomass, the use of cellulolytic enzymes for depolymerization of carbohydrate polymers into fermentable constituents, and the use of robust fermentative microorganisms for ethanol production (Soccol et al., 2010; Chandel and Singh, 2011). The cost of cellulosic ethanol is a major obstacle. However, integration of the saccharification of biomass and fermentation of released sugars into simultaneous saccharification and fermentation (SSF) has made a crucial impact on lowering ethanol production costs (Olofsson et al., 2008). The successful implementation of this technology depends on the biocatalysts used in the process. The application of thermolabile cellulases and thermotolerant ethanologens in this process may

provide “economic ethanol” with increased yield and productivity (Demirijan et al., 2001; Barnard et al., 2010).

Thermophiles produce specialized proteins known as chaperonins with inherent properties of high thermostability, denaturation, and proteolysis (Kumar and Nussinov, 2001). These proteins exhibit electrostatic bonds, disulfide bridges, and hydrophobic interactions (Kumar and Nussinov, 2001), which lead to increased thermostability. The enzymes derived from thermotolerant microorganisms are more rigid proteins than those derived from mesophiles, and thus are more stable in harsh reaction conditions. The cell membrane architecture of thermophiles reveals the presence of saturated fatty acids, which provide a hydrophobic environment for the cell and keep the cell rigid enough to live at elevated temperatures (Demirijan et al., 2001; Barnard et al., 2010). Additionally, thermophiles have been reported to have reverse DNA gyrase, which produces positive supercoils in the DNA, raising the melting point of the DNA (Haki and Rakshit, 2003). In this chapter we describe significant developments in the production of thermolabile cellulases from various microorganisms and their characteristics, including their coordinated impact on second-generation ethanol production.

10.2. THERMOPHILIC CELLULASES FOR DECONSTRUCTION OF THE PLANT CELL WALL

Temperature-resistant “extremophiles” have a special ability to survive under extreme environments (such as hot springs, volcanic areas, the deep sea, or other particular geothermal sites), due to their specific enzymes and biochemical pathways (Barnard et al., 2010; Zambare et al., 2011). It is believed that the action of enzymes derived from extremophiles (extremozymes) principally involves thermophilic enzymes that present high specificity and thus have considerable potential for many industrial applications (Averhoff and Müller, 2010). Thermostable enzymes present intrinsic activity and stability at extreme temperatures (50°C to the known upper limit of 113°C), as well as low water activity and high hydrostatic pressure. Among the enzymes produced by thermophilic microbes, glycosidases have been the most widely studied, due to their application in the polysaccharide process; their benefits include reduced risk of contamination as well as higher reaction rates due to a decrease in viscosity and an increase in the diffusion coefficient (Demirijan et al., 2001; Barnard et al., 2010).

The thermostable amylases are used extensively in the starch industry, where the enzymatic conversion of starch requires a combination of α -amylases (EC 3.2.1.1), β -amylases (EC 3.2.1.2), glucoamylases (EC 3.2.1.3), isoamylases (EC 3.2.1.68), or pullulanases (EC 3.2.1.41). The starch process involves three steps: (1) gelatinization, or dissolution of starch granules to form a viscous suspension by heating starch with water; (2) liquefaction to decrease the viscosity; and (3) saccharification to produce glucose and maltose. The thermostable enzymes are the key ingredients in liquefaction and saccharification.

In biorefinery, thermal hydrolases have been used to generate metabolizable sugars from lignocellulosic biomass. Thermostable cellulases are in demand for extended processing at elevated temperatures, particularly for converting biomass into biofuels (Turner et al., 2007). The thermostable cellulase complex comprises endoglucanases (EC 3.2.1.4), exoglucanases (EC 3.2.1.74 and EC 3.2.1.91), and β -glucosidases (EC 3.2.1.21); mediates biomass saccharification at higher temperatures; and acts to deconstruct the plant cell

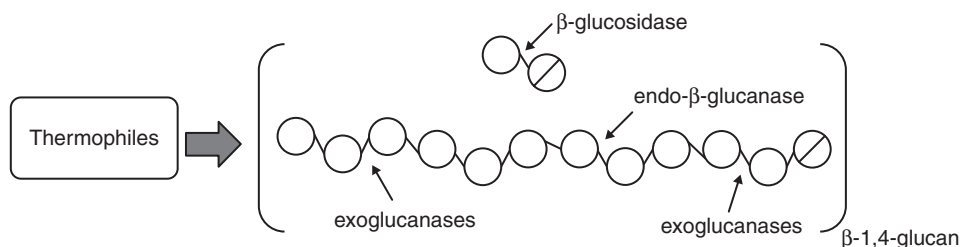


Figure 10.1. Mechanistic application of thermophilic/thermotolerant cellulase on cellulosic degradation into glucose.

wall, leading to the release of fermentable sugars from constituent polysaccharides (Maki et al., 2009). Figure 10.1 shows the mechanistic application of cellulases derived from thermophilic and thermotolerant microorganisms for the deconstruction of cellulose into glucose.

10.2.1. Thermophilic Cellulase Production and Characteristics

Thermophilic microbes have been found in natural and artificial thermal habitats, and many different sources have been used to screen for cellulolytic thermophiles (Barnard et al., 2010; Zambare et al., 2011). A cellulose-degrading bacterium capable of growth under temperatures up to 60°C was isolated from a palm oil mill industry composting system (Baharuddin et al., 2010). *Bacillus* strains capable of degrading cellulose were isolated from hot springs (Mawadza et al., 2000) and from composting (Mayende et al., 2006). Cellulolytic thermophiles can also be found in waste processes involving thermophilic aerobic digestion (Ugwuanyi et al., 2004) and in such unusual environments as manure (Lee and Blackburn, 1975).

Experiments involving cultures from thermal-pool sites enriched with the microcrystalline cellulose Avicel resulted in the screening of anaerobic bacteria cellulolytic cultures presenting carboxymethyl cellulase (CM-cellulase) activity (Sissons et al., 1987). In another case, anaerobic bacteria were found growing in water and cellulose-containing sediments at natural thermal sites at 75°C (Sharrock et al., 1982). A thermophilic microbial consortium presenting cellulolytic and xylanolytic enzymes were isolated from yard waste compost enriched with carboxymethyl cellulose (CMC) and birchwood xylan (Zambare et al., 2011). Among microorganisms, fungi such as *Sporotrichum thermophile*, *Aspergillus fumigatus*, *Thermoascus aurantiacus*, and *Humicola* spp. have been exploited as thermostable cellulase producers (De Paula et al., 1999; da Silva et al., 2005; Grigorevski-Lima et al., 2009).

Cultivation of thermophilic microorganisms has advantages for operating processes at high temperatures, as it reduces the risk of contamination and the viscosity of the medium, which enhances the substrate and gas solubility (Barnard et al., 2010). However, challenges remain in the stability of solid media and in the decomposition of complex media generally used for cultivation, and different biochemical features have been developed for efficient enzyme production by these strains (Holst et al., 1997; Synowiecki et al., 2006).

Cellulase production has been described as susceptible to catabolite repression regulation under inducible or constitutive conditions. Too much glucose or too much of another readily fermentable carbon source can repress cellulosic expression (Canevascini et al.,

1981). Production of β -glucosidase by *Sporotrichum thermophile* depended on the carbon sources used as inducers (cellulose, cellobiose, laminaribiose, and arbutin) and was essentially intracellular. The growth on cellobiose also induced CM-cellulase production (Canevascini et al., 1981). The presence of glucose and cellobiose repressed cellulase synthesis by an anaerobic thermophilic bacterium strain M7 belonging to the *Clostridium* genus, which was induced during exponential growth on cellulose and CMC as sole carbon sources, separately; maximum cellulase activity was observed in media containing cellulose (1.0%, m/w) after 72 h of incubation (Lee and Blackburn, 1975).

Extracellular endocellulases were produced by *Chaetomium thermophilum* CT2 at 50°C using the inducible carbon source microcrystalline cellulose (2%, w/v) with the addition of soluble starch (1%, w/v) and yeast extract medium (0.4%, w/v) (Li et al., 2003). The bacterium *Paenibacillus campinasensis* BL11 was able to grow over a wide temperature (25 to 60°C) and pH range, producing cellulases, xylanases, pectinases, and cyclodextrin glucanotransferase when supplied with different cellulolytic substrates (Ko et al., 2007).

Agro-waste residues rich in lignocellulose have been considered as economical alternative carbon sources (Synowiecki et al., 2006). Rice straw was used as a carbon source to induce maximal levels of cellulase production by the thermophilic fungi *Melanocarpus* sp. MTCC 3922 and *Scytalidium thermophilum* MTCC 4520 (Kaur et al., 2006). Da Silva et al. (2005) evaluated avicelase and CM-cellulase production by *Thermoascus aurantiacus* grown on different residues under solid-state fermentation, and concluded that orange bagasse, sugarcane bagasse, and sawdust were very poor substrates for enzyme production compared to green and dried grass, corn straw, and corncob, which was the best substrate inducer.

The use of sugarcane bagasse as the sole carbon source induced avicelase enzyme (EC 3.2.1.91) production by thermophilic *Geobacillus stearothermophilus* (Makky, 2009). *Thermoactinomyces* and *Bacillus* spp. presented maximal cellulase production when grown on the residues of *Brassica rapa* residues in a packed-bed reactor (Chang et al., 2009). Banana agro-waste was used as a substrate for ethanol fermentation by thermophilic and cellulolytic *Clostridium thermocellum* CT2, presenting the optimum temperature at 60°C (Reddy et al., 2010). Wheat bran and citrus pectin (1 : 1) were used in the solid-state fermentation of *Sporotrichum thermophile* to produce xylanases, pectinases, and cellulases at 45°C (Kaur and Satyanarayana, 2004).

Generally, bacterial glycoside hydrolases are often expressed in multienzyme complexes, providing increased functions and synergism. The thermophilic *Bacteroides* sp. strain P-1 presented an extracellular multienzyme complex that exhibited a high molecular mass (≥ 1400 kDa) and could affect hydrolysis of crystalline cellulose and lignocellulosic materials such as corncob, corn hull, rice straw, and sugarcane bagasse (Ponpium et al., 2000).

Purified endocellulases from thermophilic microorganisms present different characteristics and modes of action (Table 10.2). Further, evidence of synergism between thermophilic cellulases and β -glucosidases has been described exhaustively (Gaikwad et al., 1994). The hydrolytic products of CMC from the action of the cellulolytic complex produced by a marine thermophilic eubacterium *Rhodothermus marinus* comprised glucose, cellobiose, cellotriose, and a mixture of cellopentaose and larger oligosaccharides (Hreggvidsson et al., 1996). Extracellular fluid from *Chaetomium thermophile* cultures resulted in saccharification of 69% of kallar grass straw at 50°C (Latif et al., 1994).

TABLE 10.2. Properties of Some Endoglucanases Produced by Thermophilic Microorganisms

Strain	MW ^a (kDa)	pH I ^b	pH	T (°C)	Stability	K _m (mg/mL)	V _{max}	References
<i>Bacillus</i> sp. CH43	40	5.4	—	65	50°C, pH 6–10	1.5	0.93 mmol of glucose/min/mg protein	Mawadza et al., 2000
HR68	40	5.4	—	70	50°C, pH 6–8	1.7	1.70 mmol glucose/min/mg protein	Mawadza et al., 2000
<i>Rhodothermus marinus</i>	49	—	7	95	80–100°C	—	—	Hreggvidsson et al., 1996
<i>Chaetomium thermophilum</i> CT2	67.8	—	4	60	60°C, pH 4	4.6	—	Li et al., 2003
<i>Thermoascus aurantiacus</i>	78	—	4.5–5	75	—	3.9	—	Tong et al., 1980
<i>Bacillus</i> sp. KSM-S237	86	3.8	8–9	45	50°C	—	—	Hakamada et al., 1997
1139	92	—	9	50	—	0.48	—	Fukumori et al., 1985
<i>Bacillus amyloliquefaciens</i> DL-3	54	—	8	50	50–70°C, pH 8	—	—	Lee et al., 2008
<i>Caldibacillus cellulosovorans</i>	174	4.12	6.5–7	80	—	3.4	44.7 μmol/min/mg protein	Huang and Monk, 2004

^aMolecular mass.^bIsoelectric point.

A thermostable cellulase from the newly isolated *Bacillus subtilis* DR presented optimum temperature activity at 50°C and was described as retaining 70% of its maximum activity at 75°C after incubation on CMC for 30 min (Li et al., 2008). Maximum CM-cellulase activity from a *Paenibacillus* sp. strain was detected at 60°C and pH 6.5 (Wang et al., 2008). An endocellulase from *Chaetomium thermophilum* CT2 was stable at 60°C during 60 min of incubation and presented half-lives of 45 min at 70°C and 7 min at 90°C using CMC as an enzymatic activity substrate (Li et al., 2003).

An endoglucanase from *Acidothermus cellolyticus* presented a half-life of 1 h at 85°C and only 12 min at 90°C (Tucker et al., 1989). However, an endocellulase produced by the marine thermophilic eubacterium *Rhodothermus marinus* showed unusual thermostability, retaining around 50% of its activity after 3.5 h at 100°C and 80% after 16 h at 90°C using CMC as a substrate (Hreggvidsson et al., 1996). Similarly, an alkaline endo-(1,4)- β -glucanase produced by *Bacillus* spp. KSM-S237 still presented 30% of activity after heating at 100°C and pH 9.0 for 10 min (Hakamada et al., 1997). Cellobiohydrolases from *Thermotoga* spp., also active on amorphous cellulose and CMC, were the most thermostable, with a half-life of 70 min at 108°C (Ruttersmith and Daniel, 1991).

10.2.2. Alterations in Thermophiles for High-Cellulase Titers

Industrial applications of the thermostable cellulases have been strictly dependent on the availability of these molecules; the applications can be improved by designing new fermentation bioprocesses and implementing innovative bioreactors, but mainly through the use of cloning and expression of encoding genes in mesophilic hosts (Demirijan et al., 2001; Taylor et al., 2009; Barnard et al., 2010).

Isolation and screening of new thermophilic organisms have led to the identification of several novel thermostable cellulase-producing strains. However, no single enzyme is completely suitable for complete hydrolysis of cellulose (Haki and Rakshit, 2003). The protein-engineering technology has been shown to be an efficient tool for exploitation of cellulase as a catalytic function. Recent studies involving carbohydrate-binding domain evaluation and temperature dependence of cellulase activities of *Clostridium cellulolyticum* Cel9G, *Thermobifida fusca* Cel9A, and *C. thermocellum* Cel9I aimed for an ideal recombination of natural homologs to generate highly active and stable cellulases (Maki et al., 2009; Mingardon et al., 2011).

Németh et al. (2002) evaluated the action of mutations to engineer proteins with increased thermostability, using a cellulase C from *Clostridium thermocellum* as a model of thermophilic protein, and concluded that amino acid replacement was insufficient to alter the stability of thermoenzymes. Yennamalli et al. (2011) verified that thermophilic and mesophilic endoglucanases have similar structure and are related evolutionarily. Thermostability appears to be amino-acid-sequence dependent and related to strong intramolecular interactions in proportion to the greater number of salt bridges and SC–SC hydrogen bonds in thermophiles.

A cellulase gene encoding a thermostable endoglucanase from the thermophilic eubacterium *Fervidobacterium nodosum* Rt17-B1 was overexpressed in *Escherichia coli*, resulting in a highly thermostable cellulase with a half-life of 48 h at 80°C on CMC at pH 5.5 (Wang et al., 2010). A thermostable endocellulase was also purified from *B. subtilis* DR, isolated from a hot spring, and cloned into *E. coli*, resulting in a threefold increase in

production (Li et al., 2008). A β -(1,4)-endoglucanase from the thermophilic and halotolerant eubacterium *Thermoanaerobacter tengcongensis* MB4 was also cloned and expressed in *E. coli*, and maintained high activity at 75 to 80°C in the presence of substantial amounts of NaCl and KCl (Liang et al., 2011). An endo- β -(1,4)-glucanase from the thermophilic fungus *Thermoascus aurantiacus* IFO9748 was expressed on *Saccharomyces cerevisiae* and retained activity at 70°C over 20 min at pH 6 (Hong et al., 2003).

10.2.3. β -Glucosidases from Thermophilic Microorganisms

Lignocellulosic bioconversion can be improved through a synergistic action between cellulases and β -glucosidases, since the latter can hydrolyze the cellobiosaccharides released from cellulose hydrolysis (Turner et al., 2007). Solid-state cultivation using agricultural by-products as substrates has been the most common fermentative process for thermophilic β -glucosidase production (Kalogeris et al., 2003). Unlike cellulase secretion, β -glucosidase secretion appeared to be related to the later stages of fermentation. Production of β -glucosidase by *Sporotrichum thermophile* occurred only after complete depletion of cellulose from bamboo pulp used as the carbon source, and its secretion increased with hyphal autolysis (Gaikwad et al., 1994).

These enzymes have been also cloned successfully in mesophilic hosts to improve yields. A gene for a thermostable β -glucosidase produced by *Talaromyces emersonii* fungi was expressed successfully in the filamentous fungus *Trichoderma reesei*, and the enzyme presented a half-life of 62 min at 71.5°C. This β -glucosidase also showed transferase activity in the presence of high concentrations of glucose and cellobiose (Murray et al., 2004). Hardiman et al. (2010) used random drift mutagenesis, a directed evolution technique, to screen mutants that produced β -glucosidases with higher catalytic efficiency.

The characteristic properties of thermophilic β -glucosidases are summarized in Table 10.3. A β -glucosidase was purified from *Humicola insolens* mycelia and presented stability at 50°C for 1 h and a half-life of 44 min at 55°C. The purified enzyme could hydrolyze various substrates, including cellobiose, lactose, *p*-nitrophenol (NP)- β -D-glucopyranoside, *p*-NP- β -D-fucopyranoside, *p*-NP- β -D-xylopyranoside, *p*-NP- β -D-galactopyranoside, *o*-NP- β -D-galactopyranoside, and salicin (Souza et al., 2010). On the other hand, a purified intracellular β -glycoside hydrolase from ascomycete *Talaromyces thermophilus* CBS 236.58 presented both β -glucosidase and β -galactosidase activities (Nakharat and Haltrich, 2006). Some β -glucosidases produced by *Scytalidium thermophilum* showed increased activity in the presence of monosaccharides such as glucose and xylose (Zanoelo et al., 2004), while the activity of a β -glucosidase from *Humicola grisea* var. *thermoidea* was inhibited competitively by glucose (Ferreira-Filho, 1996).

10.3. ETHANOL PRODUCTION AT ELEVATED TEMPERATURES

Process integration is necessary to make the production of ethanol from cellulotics simple and cost-effective (Chandel et al., 2010a). Combining processes, such as the saccharification of biomass into simpler sugars and their simultaneous conversion into ethanol, in a single vessel (SSF) is an effective process configuration to increase yields of ethanol and reduce costs (Olofsson et al., 2008; Chandel et al., 2010a). Thermotolerant ethanologens that can grow at or above 45°C are crucial for SSF. For example, Ueno et al. (2003) reported that

TABLE 10.3. Properties of Some β -Glucosidases Produced by Thermophilic Microorganisms

Strain	MW ^a (kDa)	pH	T (°C)	Stability	K _m (mM)	V _{max}	References
<i>Humicola insolens</i>	55	6–6.5	60	50°C, pH 6	0.51 (cellobiose) 0.16 (<i>p</i> -NPG ^b)	86 U/mg protein (cellobiose) 18.1 U/mg protein (<i>p</i> -NPG)	Souza et al., 2010
<i>Scytalidium thermophilum</i>	40	6.5	60	50°C	0.29 (cellobiose) 1.61 (<i>p</i> -NPG)	13.27 U/mg protein (cellobiose) 4.12 U/mg protein (<i>p</i> -NPG)	Zanoelo et al., 2004
<i>Penicillium citrinum</i> YS40-5	72	5	70	60°C	32.17 (cellobiose) 17.59 (<i>p</i> -NPG)	72.49 U/mg protein (cellobiose) 85.93 U/mg protein (<i>p</i> -NPG)	Ng et al., 2010
<i>Termitomyces clypeatus</i>	116	5	45	60°C, pH 6–7	0.148 (<i>p</i> -NPG)	0.077 U/mg protein (<i>p</i> -NPG)	Pal et al., 2010
<i>Thermotoga maritima</i>	81	5	85		0.0039 (<i>p</i> -NPG)	—	Goyal et al., 2001
<i>Thermosascus aurantiacus</i>	120	4.5	80	70°C, pH 5	0.1137 (<i>p</i> -NPG)	—	Parry et al., 2001
Anaerobic bacterium	43	6.2	75	—	0.73 (cellobiose) 0.15 (<i>p</i> -NPG)	35.67 U/mg protein (<i>p</i> -NPG)	Patchett et al., 1987
<i>Humicola grisea</i> var. <i>thermoidea</i>	82	4–4.5	60	60°C	0.316 (<i>p</i> -NPG)	0.459 IU/mL (<i>p</i> -NPG)	Ferreira-Filho, 1996

^aMolecular mass.^b*p*-Nitrophenyl- β -glucopyranoside.

thermotolerant yeast could produce more than 6% ethanol within 24 h at 40°C. Combining processes will cumulatively save energy in large-scale ethanol production operations using thermotolerant microorganisms, which affects the economics of overall ethanol production (Ueno et al., 2003). The cooling and subsequent distillation of ethanol is equally expensive, and this cost can also be brought down by employing thermotolerant ethanologens (Sree et al., 2000; Ueno et al., 2003).

High-temperature ethanol fermentation eases the downstream product recovery, because aqueous ethanol will readily vaporize at temperatures over 50°C by applying only a mild vacuum; this facilitates the continuous distillation or “stripping” of ethanol, which could provide further savings in the energy required for product recovery (Vane et al., 2008). However, thermotolerant ethanologens generally have low ethanol tolerance. Ethanol tolerance in thermotolerant yeasts has not been reported at more than 40 g/L (Taylor et al., 2009). Low solvent tolerance in thermotolerant microorganisms is another challenging aspect.

10.3.1. Thermotolerant Microorganisms for Ethanol Production

A number of microorganisms are available that are capable of converting sugars to ethanol. The most common microorganisms are *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, *Candida shehatae*, *Pachysolen tannophilus*, recombinant *Escherichia coli*, and *Klebsiella oxytoca*, (Chandel and Singh, 2011). These microorganisms are mesophilic in nature. *S. cerevisiae* and *Z. mobilis* are able to utilize only hexose sugars, while *P. stipitis*, *C. shehatae*, and *P. tannophilus* can produce ethanol from pentose and hexose sugars but with some limitations (Chandel et al., 2010a; Chandel and Singh, 2011). Thermotolerant microorganisms have shown many advantages over their mesophilic counterparts. Several thermotolerant ethanologens (*Saccharomyces* spp., *Kluyveromyces* spp., *Candida* spp., *Geobacillus* spp., and *Thermoanaerobacter* spp., and *Clostridia* spp.) have been studied in detail (Taylor et al., 2009; Barnard et al., 2010).

Saccharomyces spp. are generally mesophilic and grow at temperatures from 15 to 35°C. They are the most explored microorganisms in ethanol production to date, but very few thermotolerant *Saccharomyces* are known. Sree et al. (2000) isolated four *Saccharomyces* isolates after mass bioprospecting: VS₁, VS₂, VS₃, and VS₄. Of the four, VS₃ had the ability to grow even at 44°C with a standard tolerance of ethanol (Sree et al., 2000; Sridhar et al., 2002; Chandel et al., 2011).

Kluyveromyces strains are the most studied thermotolerant strains for ethanol production. Banat et al. (1992) isolated thermotolerant strains of *Kluyveromyces* from distillery wastes in India. One of the *K. marxianus* strains, IMB3, has been reported to be capable of ethanol production at 45°C. Brady et al. (1994) reported that when *K. marxianus* IMB3 grew on 2% w/v glucose, the organism produced a maximum concentration of 8.5 g/L ethanol, which represented 83% of the maximum theoretical yield. Singh et al. (1998) evaluated the ethanol production performance of *K. marxianus* IMB3 in eight 60-m³ fermenters in an industrial ethanol production plant in India using sugarcane molasses; 6.0 to 7.2% w/v ethanol production was obtained, with the added advantages of eliminating cooling during fermentation and shorter fermentation periods of 20 h.

Candida spp. have also been studied for ethanol production. These yeasts are generally mesophilic; however, a few thermotolerant *Candida* spp. have been reported. McCracken and Gong (1982) isolated eight *Candida* spp. from sugarcane compost. These yeasts were

able to grow at 40°C. One yeast strain, HT4, was able to grow at 45°C and produced 4.3% w/v ethanol in 2 days from 10% w/v glucose.

***Clostridia*.** Thermophilic *Clostridia* is an interesting thermophilic obligatory anaerobic gram-positive bacterium that has shown a potential for ethanol production (Taylor et al., 2009; Barnard et al., 2010). The two best known thermophilic *Clostridia* microorganisms are *C. thermocellum* and *C. thermohydrosulfuricum*. They grow optimally at 60 to 65°C, degrading crystalline cellulose via a cellulosome (multienzyme cellulose-degrading complex). The cellulosome is embedded on the external surface of the cell membrane. It consists of endoglucanases, exoglucanases, cellobiose phosphorylases, cellodextrin phosphorylases, and β -glucosidases (Demain et al., 2005). The presence of these enzymes in this microorganism makes it a suitable candidate to be incorporated into consolidated bioprocessing.

***Thermoanaerobacter* spp.** *Thermoanaerobacter* spp. are physiologically similar to the thermophilic *Clostridia*. These microorganisms can utilize D-glucose and D-xylose for ethanol production and produce ethanol along with the lactate (Taylor et al., 2009). Shaw et al. (2008) developed a double-mutant strain of *Thermoanaerobacter* with the ability to utilize D-xylose, D-glucose, mannose, and L-arabinose for 37 g/L ethanol production. Georgieva et al. (2007) studied the effect of temperature on the ethanol tolerance of a thermophilic anaerobic ethanol producer, *Thermoanaerobacter* A10, and reported tolerance up to 4.7% v/v at 70°C. Commercial biotech companies such as Mascoma Corp. and Biogasol Corp. are engaged in developing potential thermophiles for the large-scale production of bioethanol.

***Geobacillus* sp.** Another novel microorganism, a *Geobacillus* sp., has been investigated for ethanol production (Nazina et al., 2001). *G. stearothermophilus* produced ethanol along with lactate, formate, and acetate from glucose (Taylor et al., 2009). TMO Renewables Ltd., a UK-based company, has developed modified strains from *G. stearothermophilus* for commercial ethanol production from pentose and hexose sugars.

10.3.2. Improvements in Thermophilic Ethanol Producers

Adaptation. Acclimatization of newly isolated or already available microorganisms to gradually increasing temperatures or ethanol concentrations may provide suitable thermotolerant or ethanol-tolerant strains. A number of efforts have been made to develop such strains (reviewed by Banat et al., 1998). Hack and Merchant (1995) acclimatized thermotolerant yeast *Kluyveromyces marxianus* IMB3 to different ethanol concentrations up to 80 g/L. Four isolates were selected from the continuous culture, only one of which produced a significant increase in final ethanol concentration (50 ± 0.4 g/L). Ballesteros et al. (1993) adapted *K. marxianus* LG strain to different temperatures in the range 42 to 47°C. A total of 35 new clones were obtained that dramatically improved the SSF of 10% Solka-floc substrate at 45°C when compared to the original strain, some with ethanol concentrations as high as 33 g/L. Balakumar et al. (2001) thermally adapted *S. cerevisiae* isolated at 50°C to different temperatures; its levels of ethanol production from 100 g glucose/L were 46 g/L (36 h), 38 g/L (48 h), and 26 g/L (48 h) at 40, 43, and 45°C, respectively, in a rich nutrient medium.

Mutation. Improving microorganisms by mutation is a traditional process that has been implemented successfully in modern science. The major drawback of this method is the need to generate stable product titers from mutagenic strains (Banat et al., 1998). Chemical mutagens and physical methods such as exposure to Ultraviolet (UV) radiation have been used to induce favorable mutations. The chemically or physically induced mutants are then exposed to high temperatures to make ideal strains (Wati et al. 1996). Unaldi et al. (2002) isolated alcohol-tolerant, thermotolerant, and osmotolerant yeast strains and further improved their alcohol tolerance by UV mutagenesis after exposure to UV light at intervals of 20, 30, 40, and 50 s. One of the strains showed a high alcohol tolerance, which increased from 9% v/v to 12% v/v. Sridhar et al. (2002) developed an *S. cerevisiae* strain with osmotolerance, thermotolerance, and ethanol tolerance by treating with UV radiation. Pasha et al. (2007) developed a fusant yeast followed by UV and ethidium bromide mutagenesis with an ethanol yield of 0.459 ± 0.012 g/g, productivity of 0.67 ± 0.015 g/L·h, and a fermentation efficiency of 90% from *Lantana camara* enzymatic hydrolysate.

Protoplast Fusion. Protoplast fusion enables a characteristic advantage of promoting high frequencies of genetic recombination between organisms that is genetically uncharacterized (Pasha et al., 2007). This technique is considered to be of utmost importance in yeast strains, because sexual processes are frequently not available or not effective enough for efficient transfer of genetic information (Kida et al., 1992). During the protoplast fusion, a hybrid state of microorganism develops, showing the restoration of the genome on chromosomes, which leads to further genetic recombination. Kida et al. (1992) developed fusants of thermotolerant *S. cerevisiae* hybrids. Protoplasts of thermotolerant *S. cerevisiae* VS₃ and mesophilic, xylose-utilizing *Candida shehatae* were fused by electrofusion (Pasha et al., 2007). The fusants were selected based on their growth at 42°C and ability to utilize xylose. The mutant fusant CP11 was found to be stable and showed an ethanol yield of 0.459 ± 0.012 g/g, a productivity of 0.67 ± 0.15 g/L·h, and a fermentation efficiency of 90%.

Intergeneric fusants were obtained by protoplast fusion between the thermotolerant yeast *K. marxianus* and the starch-assimilating yeast *Schwanniomyces occidentalis*. Two thermotolerant fusants were obtained at 40°C that showed ethanol production and α -amylase (Leelavatcharamas et al., 2006). The fusants developed produced alcohol (7.4%) at 45°C. Krishnamoorthy et al. (2010) developed an intergeneric protoplast fusant of *S. cerevisiae* and *K. marxianus* that produced a high level of ethanol (12.5%, 18.09 g/L of biomass) after 72 h of fermentation.

Thermotolerance and Genetic Engineering. A strong “degeneration” in selective strains can occur upon the storage of conidial material; therefore, molecular-biology-based modern genetics and protein engineering have put forth new avenues to create genetically engineered microorganisms (GEMs) that can function as “booster biocatalysts” (Chandel and Singh, 2011). Despite the development of numerous industrial recombinant yeasts, thermotolerant GEMs (TGEMs) have not been used commercially for ethanol production. TGEMs can convert pentose and hexose sugars into ethanol at high temperatures, improving cellulosic ethanol production efficiency and reducing the cost of production (Alper et al., 2006). These strains can be incorporated into SSF, making the overall process simple, integrated, and economical (Olofsson et al., 2008).

In the past, different molecular biology approaches have been taken to construct or develop suitable thermotolerant strains (Turner et al., 2009). *S. cerevisiae* is the most popular strain targeted for genetic manipulation. Abdel-Banat et al. (2010) developed a mechanism of random integration in *K. marxianus* DMKU3-1042, in which the *KU70* gene involved in the nonhomologous end-joining (NHEJ) pathway was deleted. The results showed a highly efficient NHEJ pathway in *K. marxianus*, which can be altered with random gene disruption techniques, such as transposon mutagenesis and plasmid-free gene manipulations. Nonklang et al. (2008) developed the *K. marxianus* DMKU3-1042 strain for cost-effective bioethanol production and as a host for transformation with linear DNA using *S. cerevisiae*-based molecular genetics tools. The strain developed showed better growth and ethanol production than *S. cerevisiae* at 45°C. This strain also utilized cellobiose, xylose, xylitol, arabinose, glycerol, and lactose. Kitagawa et al. (2010) developed a thermotolerant strain of *Issatchenkia orientalis* for use in consolidated bioprocessing; expression of β -glucosidase from *Aspergillus aculeatus* could be achieved with *I. orientalis*, demonstrating successful heterologous gene expression in *I. orientalis*, showing the conversion of cellobiose into ethanol. The transformant produced 29 g/L of ethanol in 72 h at 40°C from 100 g/L of microcrystalline cellulose. Jeffries and Jin (2000) deciphered the mechanism of thermal and ethanol tolerance in yeasts by synthesizing trehalose and heat-shock proteins (HSPs), which stabilize and repair denatured proteins. The increased thermotolerance in cells is probably due to trehalose synthesis and the induction of HSPs. Cytoplasmic and mitochondrial superoxide dismutase can destroy oxidative radicals and thereby maintain cell viability. Ryabova et al. (2003) developed wild-type strains of the thermotolerant methylotrophic yeast *Hansenula polymorpha* for increased ethanol production from glucose, cellobiose, and xylose at high temperatures.

10.3.3. Process Development and Thermophiles

To achieve the desired yield of ethanol from thermotolerant and thermophilic microorganisms, methods need to be improved. A suitable microorganism should be able to survive on a wide range of complex substrates at higher levels of carbohydrate with little or no toxicity of the product formed. Routinely applied methods such as immobilizing cells in repeated batch fermentation or in continuous fermentation, adaptation of microorganisms to high temperatures, recycling of free and immobilized cells in fermentation, and coculturing of microorganisms have shown promising outcomes (Banat et al., 1998; Singh et al., 1998; Chandel and Singh, 2011; Kumar et al., 2011). Abdel-Banat et al. (2010) demonstrated that a 5°C increase in fermentation temperature can greatly affect the cost of fuel ethanol production. They also proposed that thermotolerant mesophilic microorganisms have considerable potential for the development of future fermentation technologies.

Ethanol production by both thermophilic and thermotolerant microorganisms has been studied broadly under various types of fermentation, including batch, fed-batch, and continuous fermentation (reviewed by Banat et al., 1998; Taylor et al., 2009; Barnard et al., 2010). In batch fermentations, thermotolerant yeasts of the genera *Saccharomyces* and *Kluyveromyces* have produced appreciable ethanol concentrations, varying between 4 and 8% w/v (Ballesteros et al., 1993; Brady et al., 1994; Banat et al., 1998). Table 10.4 summarizes the ethanol production profiles of thermotolerant microorganisms with various raw materials and cultivation conditions.

TABLE 10.4. Ethanol Production Profile from Various Thermotolerant Microorganisms Grown on Various Types of Substrates at High Temperature

Microorganisms	Fermentation Substrates	Cultivation Conditions and Temperature (°C)	Ethanol Production (g/g or g/L)	References
<i>Saccharomyces cerevisiae</i> VS ₃	<i>Saccharum spontaneum</i> enzymatic hydrolysate	Repeated batch cultivation, 42 ± 0.5	22.85 ± 0.44 g/L, yield, 0.45 ± 0.04 g/g	Chandel et al., 2009
<i>S. cerevisiae</i> VS ₃ + <i>Pichia stipitis</i> NCIM 3498	<i>Saccharum spontaneum</i> acid hydrolysate	Separate hydrolysis and fermentation (SHF), 30	15.0 ± 0.92 yield, 0.49 ± 0.02 g/g	Chandel et al., 2011
<i>S. cerevisiae</i> IR2-9a	Bleached kraft pulp	Simultaneous saccharification and fermentation (SSF), 40	28 g/L (62% of the theoretical ethanol yield)	Araque et al., 2008
<i>S. cerevisiae</i> VS ₃ + <i>Bacillus</i> sp. VB ₉	Sweet sorghum grains	Solid-state fermentation (SSF), 42	3.7 g ethanol/100 g of substrate	Sree et al., 2000
<i>Debaromyces hansenii</i>	Wheat bran hemicellulose	SSF, 40	9.5 g/L	Menon et al., 2010
Recombinant <i>Klebsiella oxytoca</i> P2	Microcrystalline cellulose	SSF, 37	33 g/L	Golias et al., 2002
<i>Issatchenkia orientalis</i> IPE 100	Steam-exploded cornstalk enzymatic hydrolysate	SHF, 42	93.8% of theoretical yield and 0.91 g/L·h of productivity	Kwon et al., 2011
<i>Kluyveromyces marxianus</i> IMB3	Hydrothermolysis treated switch grass	SSF, 45	32, 82% yield	Pessani et al., 2011
<i>Kluyveromyces marxianus</i> DMKU 3-1042	Sugarcane juice	Batch fermentation, 37	8.7 w/v, 77.5% yield	Limtong et al., 2007
<i>Pichia kudriavzevii</i>	Sugarcane juice	Recycling of cells, batch cultivation, 40	71.9 g/L, 4.0 g/L·h	Dhaliwal et al., 2011
<i>Kluyveromyces marxianus</i> CECT 10875	Wheat straw	Fed-batch SSF, 42	36.2 g/L	Tomás-Pejó et al., 2009

In batch fermentation, microorganisms can survive efficiently at higher substrate concentrations and provide an increased yield of ethanol. However, in the case of thermotolerant yeasts, during batch fermentation a combined effect of high substrate concentration, high temperature, and high ethanol concentration can inhibit growth (D'Amore et al., 1990; Banat et al., 1998). Fed-batch fermentation could be an effective strategy to overcome these conditions. During fed-batch operations, high concentrations of substrate are added gradually, which makes microorganisms susceptible at high sugar levels (Xu et al., 1996).

Continuous fermentation is an ideal strategy for ethanol production from thermophiles and thermotolerant microorganisms. The continuous process has shown substantial improvements in ethanol production efficiency and product quality, including higher productivity; lower operating costs; reduced product losses; and environmental advantages (Verbelen et al., 2006; Kumar et al., 2011). The continuous process could be an effective strategy to overcome the inhibitors present in lignocellulose hydrolysates. During continuous fermentation, a steady state is maintained in the reactor vessel for a period of time (Verbelen et al., 2006), which eliminates adverse effects due to high sugar concentration and ethanol accumulation. A number of inorganic and organic supports have been used for cell immobilization for continuous ethanol fermentation (reviewed by Kumar et al., 2011).

Mixed-culture cultivation has been recognized as another ideal approach to expedite the maximum conversion of available fermentable carbohydrates (pentose and hexose sugars) into ethanol. In an attempt to maximize the utilization of carbon biomass, *C. thermocellum* has been cocultured with other thermophilic strains, including *Clostridia thermosaccharolyticum*, *C. thermohydrosulfuricum*, *T. ethanolicus*, *G. stearothermophilus*, and *Thermoanaerobacter brockii* (Taylor et al., 2009). Chandel et al. (2010b) observed maximum ethanol production of 17.73 ± 0.25 g/L (productivity 0.246 ± 0.02 g/L·h) from *Saccharum spontaneum* enzymatic hydrolysate under SSF using a coculture of mesophilic *Pichia stipitis* NCIM3498 and thermotolerant *S. cerevisiae* VS₃.

10.4. FUTURE PERSPECTIVES AND CHALLENGES

Rapid industrial development, increased energy demand, the unstable supply of gasoline, regular hikes in gasoline prices, and environmental pollution concerns have made it necessary to find environmentally sustainable and renewable energy sources at a lower cost per gallon (Graham-Rowe, 2011). Bioethanol is one of the most attractive sustainable energy sources to fuel transportation, and it is being implemented as an energy alternative throughout the world. The demand for ethanol production in the United States and Brazil is currently being met using crops such as corn and sugarcane. Considering the food and feed value of these crops and the likelihood of future shortages, utilization of a cheaper substrate such as lignocellulose could make bioethanol more competitive with fossil fuel (Graham-Rowe, 2011). The processing and utilization of lignocellulosic substrate is complex, differing in many aspects from crop-based ethanol production. Efficient ethanol production processes and cheap substrates are needed. The cost of ethanol production depends directly on the cost of cellulytic enzyme cocktails (Chandel et al., 2010a). The need of the hour is to develop cheap and efficient production systems for thermophilic cellulases that can be applied for biomass saccharification and the conversion of released sugars into ethanol under a consolidated process technology (Taylor et al., 2009).

Improved bioprospecting techniques, optimization of fermentation parameters and media formulations, and systems biology-based genomics, proteomics, and metabolomics

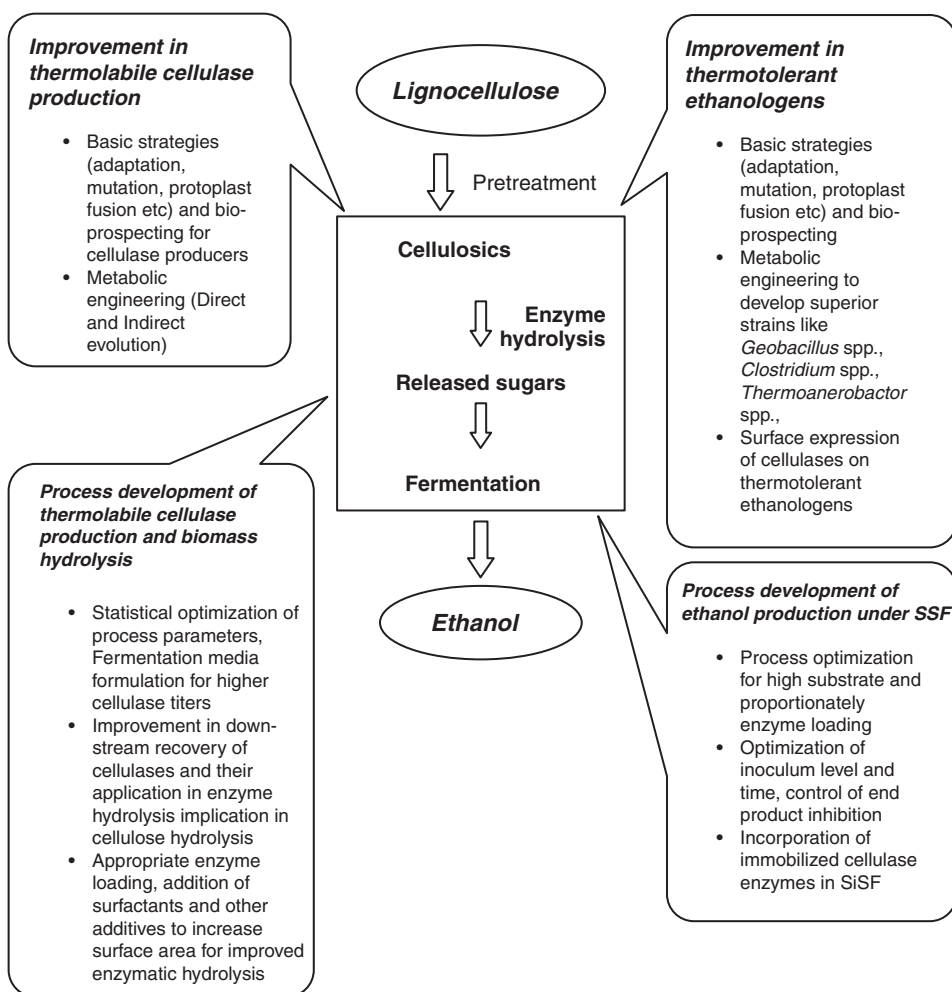


Figure 10.2. Procedural implication of simultaneous saccharification and fermentation for bioethanol production, highlighting the future needs for development.

approaches have the potential to trigger improved ethanol production from thermophilic cellulase obtained from thermophilic microorganisms. One of the biggest challenges is scaling up the process in a cost-effective manner. The use of thermophilic and thermotolerant microorganisms has several advantages that make the overall process more economical and speed up production (Banat et al., 1998; Barnard et al., 2010; Zambare et al., 2011). Figure 10.2 presents selective methods for obtaining high titers of thermophilic cellulase, production optimization, integrated application for improved cellulosic hydrolysates, and the subsequent production of ethanol by SiSF. The benefits associated with working at high temperatures, such as energy savings, rate of product formation, minimization of contamination, and process simplification, suggest that thermophilic ethanologens have the potential to overcome the commercial barriers to second-generation biofuels and make lignocellulosic ethanol a reality in the near future.

10.5. CONCLUSIONS

Over the past few decades, there has been a continuous thrust to develop sustainable, renewable alternatives to gasoline. Currently, ethanol is the most feasible alternative energy source. Today, ethanol for blending with gasoline is produced from sugarcane juice, corn grains, and other food- and feed-based raw materials, which is not a long-term solution, due to the increased demand for food and feed around the globe. Ethanol produced from nonfood materials such as lignocellulose is definitely a more feasible solution, due to their enormous availability in nature; the production of lignocellulosic ethanol using cellulases is inevitable. Thermostable cellulases can play a key role in biomass saccharification, producing large amounts of sugars that are converted into ethanol by thermophilic and thermotolerant ethanologens. Integration of these two steps in one vessel (i.e., simultaneous saccharification and fermentation) is pivotal to the production of economical second-generation ethanol.

Acknowledgments

The authors are grateful to BIOEN-FAPESP and CNPq for their financial support.

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ECOFRIENDLY ASPECTS OF THE USE OF EXTREMOPHILIC ENZYMES IN TEXTILE SUBSTRATES

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11.1. INTRODUCTION

Biotechnology is the application of living organisms and their components to industrial products and processes. In 1981 the European Federation of Biotechnology defined *biotechnology* as the “Integrated use of Biochemistry, Microbiology and Chemical Engineering in order to achieve the technological application of the capacities of microbes and cultured tissue cells.”

Rapid developments in the field of genetic engineering have given a new impetus to biotechnology. This introduces the possibility of tailoring organisms to optimize the production of established or novel metabolites of commercial importance and of transferring genetic material (genes) from one organism to another.

Biotechnology also offers the potential for new industrial processes that require less energy and are based on renewable raw materials. It is important to note that biotechnology is not just concerned with biology but is a truly interdisciplinary subject involving the integration of natural and engineering sciences.

Defining the scope of biotechnology is not easy because it overlaps with so many industries, notably the chemical or food industries, but biotechnology has also found many applications in the textile industry, especially in the preparation of biopolymers, textile wet processing, and effluent management (Rehm and Gerald, 2001).

The use of enzymes as biotransformation catalysts is well established and is reported in numerous texts and reviews. The majority of enzymes used to date, however, have been obtained from mesophilic organisms, and despite their many advantages, use of these enzymes is restricted because of their limited stability to extremes of temperature, pH, ionic strength, and so on. On the other hand, extremophiles are a source of enzymes (extremozymes) with extreme stability toward a wide range of temperature, from -2 to 15°C and 60 to 110°C , ionic strength (2 to 5 M NaCl), and pH (less than 4 and greater than 9). The application of these enzymes as biocatalysts in chemical wet processing of textiles is attractive because they are stable and active under conditions that were previously regarded as being incompatible with biological materials. Some of these extremophilic enzymes have novel metabolic pathways and so might serve as a source of enzymes with vital activities and applications in the textile industry (Karmakar, 1999; Gupta, 2001; Warke and Chandratre, 2003; Kumar et al., 2011).

11.2. BIOPOLYMERIC FIBERS

Natural fibers, which are usually biodegradable in nature, have been used by humankind for apparel and allied textile uses since ancient times. Today, synthetic fibers are widely used as textile materials, due to their ease of production and economic aspects, but because of their nonbiodegradability, they pose a threat to the environment. Research work is going on in various parts of the world to produce synthetic biopolymers.

Natural biopolymers include cotton, jute, silk, ramie, and linen, among others. Jute and silk fibers are the best examples of biopolymers. Jute fibers are composed primarily of the plant materials cellulose (major component of plant fiber) and lignin (major component of wood fiber). Jute is 100% biodegradable and recyclable and thus environmentally friendly. It is a natural fiber with a golden and silky shine and hence is called the *golden fiber*. It is the cheapest vegetable fiber, procured from the bast or skin of the plant's stem. It is the second most important vegetable fiber after cotton in terms of use, global consumption, production, and availability.

Silk fiber (technical name β -keratin) is made up of the amino acids Gly-Ser-Gly-Ala-Gly and forms β -pleated sheets. The main amino acid is Gly (glycine). The three amino acids bond into a long chain that is repeated, forming β -pleated sheets. Silk is a natural biopolymer found in spiders and silkworms. Dextran is also a natural biopolymer, obtained by the fermentation of sucrose by *Leuconostoc mesenteroides* or related species of bacteria. It has been developed as a fibrous nonwoven material for specialty end uses. Enzymes (dextranases) from molds such as *Penicillium* and *Verticillium* have been shown to degrade dextran into low-molecular-mass sugars such as glucose and isomaltose (Ebert and Schenk, 1968). Dextran is therefore biodegradable, and dextran by-products are absorbed readily into the natural environment.

On the other hand, synthetic polymers or plastics are produced from crude oil. As the world supply of oil gradually diminishes and the price continues to increase, it will be essential to produce polymers from other sources. Moreover, these synthetic polymers are

nonbiodegradable, which causes a tremendous threat to the environment. One solution to these problems is the use of biopolymers produced from renewable resources. The Lenzing Company has succeeded in soaking normal Tencel with the biopolymer chitosan for the development of the Tencel C fiber, which has completely new applications, from the medical sector to the cosmetics industry (www.fashion.bg, 2011).

Poly(hydroxyalkanoate) (PHA) represents a family of biopolyesters synthesized by a variety of microorganisms. They are bioplastics and their properties are similar to those of many petroleum-based thermoplastics and elastomeric materials, but unlike most petroleum plastics, PHAs are sustainable, biodegradable, and biocompatible. Novel fiber-forming biopolymers are also being manufactured using large-scale fermentation equipment. One such fiber is poly(hydroxybutyrate) (PHB), which has been developed by Zeneca Bioproducts under the trade name Biopol. Biocompatibility and biodegradability make PHB fibers ideally suited for surgical use; sutures made from PHB get slowly degraded by the body's enzymes (Bonartsev et al., 2009). Recently, production of Biopol from plants is being considered. The Zeneca Seeds company is experimenting with a genetically engineered variety of grape that can synthesize Biopol. Wound dressings based on calcium alginate fibers have been developed by Caurtauld and marketed under the trade name Sorbsan. This is a polysaccharide extracted from brown seaweeds.

11.3. EXTREMOPHILIC ENZYMES AND THEIR USE IN THE TEXTILE INDUSTRY

Enzymes are biomolecules that catalyze (i.e., increase the rates of) chemical reactions. Most enzyme reaction rates are millions of times faster than those of comparable uncatalyzed reactions. The activity of the enzymes can be affected by other molecules. Inhibitors are molecules that decrease enzyme activity, whereas activators are molecules that increase the activity of enzymes. Their activity is also affected by temperature, the chemical environment (e.g., pH) as well the concentration of substrate (textile material).

Extremophilic enzymes are very specific, because both the enzyme and the substrate possess specific complementary geometric shapes that fit exactly into one another. This is often referred to as the “lock and key” model. The enzyme reactions are taking place in two stages. In the first step, the substrate binds reversibly to the enzyme, forming an enzyme–substrate complex sometimes called the *Michaelis complex*. In the second step, the enzyme catalyzes the chemical step in the reaction and releases the product. This model explains the enzyme specificity, but it fails to explain the stabilization of the transition state that enzymes achieve. The enzymes are classified mainly into the following six categories on the basis of the functions they perform:

1. *Oxidoreductases*: catalyze oxidation–reduction reactions such as oxidases (which oxidize) and reductases (which reduce)
2. *Transferases*: transfer a functional group: for example, transaminases (which transfer amino groups) and kinases (which transfer phosphate groups)
3. *Hydrolases*: catalyze the hydrolysis of various bonds: for example, proteases (which hydrolyze peptide bonds) and lipases (which hydrolyze lipid ester bonds)

TABLE 11.1. Various Applications of Enzymophilic Enzymes in Textile Wet Processing

No.	Enzyme	Extremophile	Enzyme Substrate	Textile Application
1	α -Amylase	Acidophile, thermophile, psychrophile	Starch	Desizing of cotton
2	Catalase	Thermo-alkaliphile	Hydrogen peroxide	Removal of hydrogen peroxide after bleaching
3	Cellulase	Acidophile, thermophile, psychrophile	Cellulose	1. Biostoning of denims 2. Biofinishing and giving peach skin effect to lyocell, viscose, etc. 3. Carbonization of wool
4	Pectinase	Thermophile	Pectin	Bioscouring of cotton (removal of pectin from cotton)
5	Protease	Thermophile, psychrophile halophile, alkaliphile	Proteins	1. Shrinkage reduction; improved dyeability, hand, and whiteness to wool 2. Degumming of silk and production of sand wash effect
6	Esterases	Thermophile	Ethylene glycol dibenzyl ester, terephthalic acid diethyl ester	Depilling and improvement of dyeing of polyester fibers
7	Laccase	Hyperthermophile	Indigo	Decolorization of indigo
8	Nitril hydralase and nitrilase	Thermophile	Nitrile group	Improvement of dyeing of acrylic fibers

4. *Lyases*: cleave various bonds by means other than hydrolysis and oxidation, such as carboxylases (which add CO₂) and hydrolases (which add water)
5. *Isomerases*: catalyze isomerization changes within a single molecule
6. *Ligases*: join two molecules with covalent bonds

Extremophilic enzymes have been used as process aids in the textile industry for decades. Desizing with amylases was the first applications of enzymes. With the development of the application of cellulases for treatment of denim and other cotton fabrics, the textile industry became a focus area for enzyme manufacturers. Today, approximately 80% of all denim is treated with cellulases. In addition, about 10% of all other finishing of cellulose materials is now performed using cellulases. An overview of the application of cellulases and other enzymes studied or used for textiles is provided in Table 11.1.

11.3.1. Amylases

Amylases are widely used as desizing agents to remove starch from fabrics after weaving. Enzymes capable of hydrolyzing starch are α -amylases, β -amylases, amyloglucosidase

(glucoamylase), and isoamylase. Amylases follow an acid–base mechanism for the hydrolysis of starch, which involves two acidic amino acids such as aspartic acid and/or glutamic acid together with a basic amino residue (i.e., histidine). Since amylases have been naturally designed to act on an insoluble substrate, most amylases have an extra substrate-binding domain. This brings the catalytic domain into the close vicinity of the target substrate, enhancing the catalytic performance of the enzyme.

Most commercial amylases used are crude mixtures of thermostable enzymes of bacterial origin. Amylases are activated by Ca^{2+} ions, and it is known that these enzymes perform well in hard water rich in bivalent ions. The presence of Ca^{2+} enhances the enzymatic reaction up to a certain level and is believed to stabilize catalytic sites through structural organization. The presence of calcium ions is a very important feature of bacterial thermostable amylases (stable up to 110°C); they enhance the stability of the enzymes by cross-linking the folded structure (Cegarra, 1996).

11.3.2. Cellulases

Cellulases are used in textile processing mainly for stone washing, biopolishing, and carbonization of wool. They are also a part of detergent formulations to enhance detergency, which improves brightness and removes microfibrils. Cellulose is a polysaccharide that can be hydrolyzed enzymatically by synergistic action of endo-1,4-glucanases, cellobiohydrolases, and α -glucosidases. It has been suggested that endoglucanases randomly cleave cellulose into smaller fragments, generating new ends which are then hydrolyzed endwise by the action of cellobiohydrolases.

There are two types of cellulases, acid and neutral. In chemical terms, cellulase enzyme has two regions. The globular head is the core catalytic region, whereas the tail is a cellulose-binding domain (CBD). However, the effectiveness of the catalytic core (to hydrolyze glycosidic linkages) depends greatly on adsorption by the CBD tail. When tightly bound to the substrate, certain endoglucanases disturb the crystalline structure and induce defibrillation, a result of the mechanochemical effect by the binding domains (Kochavi et al., 1990; Tyndall, 1990; Fornelli, 1992; Byrne and Rigby, 1995; Rahkamo et al., 1996, 1998).

A cellulase enzyme has various activities: the endoglucanases EG I and EG II, CBH III (exoglucanase or cellobiohydrolase), and EG III and EG IV. These activities have varying effects on the crystalline and amorphous regions of cellulose.

11.3.3. Pectinases

Pectin-degrading enzymes have received much attention for their use in the pretreatment of textile fabrics (bioscouring) prior to dyeing. The removal of pectin components from the cotton cell wall is claimed to improve fiber hydrophilicity, to facilitate dye penetration, and to contribute to substantial water savings compared to the traditional alkaline scouring process (Takagishi et al., 2001).

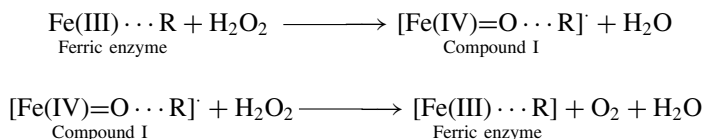
In nature, three major classes of enzymes are involved in the degradation of pectins:

1. *Pectin esterases* catalyze the deesterification of poly(methyl galacturonate), forming pectic acid (polygalacturonate).

2. *Polygalacturonases* cleave α -(1,4)-glycosidic linkages in polygalacturonate and can be divided into two groups according to their mode of action on the polymer:
 - a. Endopolygalacturonases hydrolyze randomly within pectic acid.
 - b. Exopolygalacturonases cleave in a sequential fashion, generally from the nonreducing end of the pectin chain.
3. *Protopectinases* are enzymes that solubilize pectin from protopectin. Examples are endoarabinases, pectate lyases, and polygalacturonases.

11.3.4. Catalases

Catalases are used in textile processing for the removal of residual hydrogen peroxide after bleaching. Catalases convert hydrogen peroxide into water and oxygen, showing first-order kinetics (Schmidt, 1995; Jensen, 2000). This loop reaction starts by oxidation of the catalase to compound I by one molecule of hydrogen peroxide, yielding water and regeneration via production of oxygen from the second molecule of H_2O_2 .



11.3.5. Proteases

Proteases are important components of detergent formulations for removing protein stains (e.g., egg, blood) from various textiles (Hsieh and Cram, 1999; Buchert et al., 2000). Additionally, proteases have a useful potential in silk and wool processing (Chikkodi, 1996; Miettinen-Oinonen et al., 2001). Proteolytic enzymes (or proteases) catalyze the hydrolysis of certain peptide bonds in protein molecules. Proteases attack proteins via two modes, yielding different products:

1. Endoproteases attack peptide bonds in the interior of the peptide chain, yielding smaller polypeptides and peptides.
2. Exoproteases act by cleaving off single amino acids from either end of the peptide chain. Exoproteases are specific according to which end of the protein chain they attack: carboxypeptidases if they attack the end with a free carboxylic acid (C-terminus), aminopeptidases if they attack the free amino end group (N-terminus).

Different proteases have different specificity in regard to which peptide bonds are broken. This depends on the nature of the adjacent amino acid side chains and the spatial arrangement of the polypeptide chain in the protein structure.

11.3.6. Esterases

Esterases have been suggested as being useful components of detergent formulations to remove lipid-based stains from textiles, while some esterases have been claimed to hydrolyze

polyester (Andersen et al., 1998). Esterases hydrolyze ester bonds, and their classification is based on the type of ester bond hydrolyzed. Esterases with applications in textile processing include:

- Carboxylesterases, which hydrolyze carboxylic esters, yielding the corresponding alcohol and carboxyl anion
- Arylesterases, which hydrolyze phenyl acetate to phenol and acetate
- Triacylglycerolesterases, which hydrolyze triacylglycerol, giving a diacyl glycerol and a fatty acid anion

11.3.7. Laccases

Laccases in combination with redox mediators are used in textile processing to bleach denim fabrics, that is, to decolorize indigo. Research efforts have been made to use laccase as a bleaching and/or oxidative coupling agent for dyeing animal fibers and human hair. Laccases are unspecific oxidoreductases that catalyze the removal of a hydrogen atom from the hydroxyl group of *ortho*- and *para*-substituted mono- and polyphenolic substrates and from aromatic amines by one-electron abstraction while the co-substrate oxygen is reduced, yielding water. Free radicals formed in this reaction from the substrates are capable of undergoing further depolymerization, repolymerization, demethylation, or quinone formation (Shelke, 2001; Ramakrishnan, 2002).

11.4. UTILIZATION OF EXTREMOPHILIC ENZYMES IN TEXTILE WET PROCESSING

Extremophilic enzymes find applications in various processes, ranging from fiber preparation to finishing and subsequent laundering of textiles.

- Fiber and fabric preparation: retting of bast fibers, carbonization of wool, degumming of silk, etc.
- Pretreatment: desizing of cotton, scouring, bleaching, etc.
- Dyeing and printing of textiles
- Finishing: biopolishing of silk, wool, jute, polyester, lyocell, etc.
- Washing and laundering with biodetergents

11.4.1. Fiber Preparation

Retting of Bast fibers. Bast fibers, such as flax, hemp, jute, and kenaf, are composed of cellulose (over 50%), hemicelluloses, lignin, pectins, fats, and waxes. The bast fibers are situated in the stem between the cortex and the hollow central wood, as shown in Figure 11.1, and are not easily separated from the other plant tissues unless some decomposition of the stem takes place. This process of decomposition is called retting. By this process, the matrix components, mainly pectin and lignin, are removed and the fibers are separated.

Traditionally, two types of retting have predominated, dew retting and water retting, which rely on indigenous soil fungi or on bacteria in an aqueous environment, respectively.

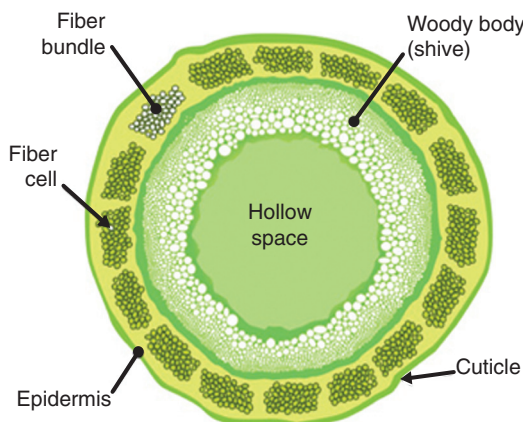


Figure 11.1. Cross section of a typical bast fiber.

Chemical retting (i.e., the use of acids, bases, surfactants, and chelators) has also been tested for the separation of fibers from nonfibrous tissues. Various attempts have been made since the late 1970s to introduce more rapid and controllable enzyme retting processes to replace the conventional dew- and water-retting processes. Several different enzymes (e.g., pectinases, cellulases, hemicellulases) have been tested (Ramakrishnan, 2002). Pretreatment of flax fibers with sulfur dioxide gas brings about sufficient breakdown of the woody straw material to speed up enzyme retting while preventing excessive bacterial or fungal deterioration of the fiber. Despite the progress made during this period, no enzymatic retting method has yet been commercialized.

Recently, the National Research Council Biotechnology Research Institute (NRC-BRI) in Montreal has been working to develop energy-efficient, environmentally acceptable ways to transform hemp and flax into high-quality fibers. The first challenge is to develop a reliable, cost-efficient, and ecofriendly way to recover natural fibers from straw. Although field and water retting also involve breakdown by microorganisms of the woody stems of the fibers over time, these traditional processes are slow, uncontrolled, and raise environmental concerns. The researchers are trying to develop an enzyme that will do a better job of removing the plant cuticle (skin) without digesting the fiber inside so as to give not only high-quality fibers but also to produce fibers with improved surface characteristics. The NRC has already filed for a patent on its genetically modified pectate *lyase* and will seek to patent other enzymes in the future. The ultimate goal is to license these enzymes to Canadian entrepreneurs to help bring bioprocessing into the mainstream for fiber production.

Carbonization of Wool. Raw wool contains seeds and other pieces of vegetable matter, technically referred to as *burr*. Much of this may be removed in scouring and in combing. Combing is not used in woolen manufacture, and vegetable matter is only partially removed in carding. Even in some worsted processes, a small amount of residual vegetable material is present in the fabric, and in such cases carbonizing is essential to remove the residues (Elisabeth and Hartwig, 1995; Doshi and Shelke, 2001).

The scoured wool fabric is padded, either in rope or open width form, with liquor containing dilute sulfuric acid (5 to 7 wt%) at approximately 65% wet pickup and is dried

at 65 to 90°C to concentrate the acid. Baking at 125°C for 1 min chars the cellulosic material. The charred vegetable material is brittle and easily crushed on passing through rollers. It can be removed as dust during subsequent mechanical working. After carbonizing, the wool fabric should be rinsed and neutralized by thorough washing. Such neutralization should be carried out immediately after baking; otherwise, fabric damage will occur during storage of wool in such an acidic state. It is convenient to neutralize prior to dyeing, but uneven neutralization leads to uneven dyeing.

The carbonization of wool with inorganic acids may cause some degradation of the fibers. Replacement of carbonization by the use of such enzymes as cellulases, lignases, hydrolases, lyases, and oxidoreductases has been reported in the literature. The main advantages of enzymatic carbonizing are reduced wool fiber damage, effluent load, and energy consumption. Natural soiling of wool, such as by vegetable matter and skin flakes, can also be modified enzymatically. Burr removal becomes easier after lubricating wool with cellulases, due to weakening of cohesion between the burr and the wool fiber. Lignin of the burrs in wool can be degraded by the use of lignin peroxidases.

A biochemical alternative, using a complex combination of enzymes, to the chemical process of carbonizing with sulfuric acid has also been reported. The amount of sulfuric acid required for carbonization can be reduced by the action of cellulolytic and pectinolytic enzymes.

Degumming of Silk. The silk filament spun by the silkworm *Bombyx mori* is composed of two fibroin filaments held together by a cementing layer of sericin. Fibroin and sericin account for about 75% and 25% of raw silk, respectively. The degumming process consists of removing the sericin layer prior to dyeing or printing and finishing to get bright, lustrous fabrics and garments (Fig. 11.2).

The degumming of silk has conventionally been carried out under alkaline conditions at a pH of 10 to 11 near boiling. In recent years, two new processes have emerged: high temperature–high pressure degumming and enzymatic degumming. *High temperature–high pressure degumming* requires special pressured equipment and is energy-intensive. *Enzymatic degumming* is emerging as an ecofriendly fiber-gentle process using proteolytic enzymes (protease) which are effective under alkaline, neutral, and acidic conditions (Annamaria et al., 1998; Nargunamani and Selvakumar, 2002; Cavaco-Paulo and Gilbitz, 2003; Freddi et al., 2003; Yu-Qing et al., 2004; Shen, 2010). With local availability of the enzymes at a reasonable price, this process has a commercial potential in India. Being large



Figure 11.2. Raw silk and degummed silk. (See insert for color representation of the figure.)



Figure 11.3. Degumming of silk with enzyme.

molecules, enzymes do not penetrate the interstices of the fabric and hence are suitable for yarn degumming only (Fig. 11.3). Critical control of the pH and temperature is required to realize the full potential of the enzymes, requiring use of sophisticated machinery such as the Mezzera yarn-dyeing machine. Since most of the enzymes are effective at a comparatively low temperature of about 60°C, they are less energy intensive.

There are a number of advantages of degumming silk with protease extremophilic enzymes:

- The removal of sericin (gum) from silk yarn with protease, which is milder than a soap or alkaline treatment, does not cause any damage to the precious fiber.
- Protease enzymes are also used for producing sand-washed effects on silk garments.
- Treatment of silk–cellulosic blends with protease enzyme is claimed to produce some unique effects.
- The treatment saves expenses in terms of water, energy, chemicals, and effluent treatment.
- Proteases effectively remove the gum (sericin) from silk fabrics regardless of the medium.
- Almost complete sericin removal has been obtained when raw silk fabric was the substrate.
- Statistical approaches have helped to enhance product yield and reduce the cost of production, thereby making the fermentation process economical and cost-effective.

11.4.2. Fabric Preparation

The greatest number of enzymatic treatments has been applied to industrial processing of cellulosic fibers in order to obtain new finishing effects or to replace the harsh chemicals used in conventional cotton processes. Conventional processing of cotton includes several

chemical steps performed during wet process stages. Enzymatic treatments of cotton, subdivided into the three categories biopreparation, biofinishing, and biostoning, can be included in these wet processes and can be performed in already existing equipment.

Desizing of Cotton. The application of a sizing agent to warp yarns prior to weaving is essential for high weaving efficiency in the production of most fabrics because warp cotton yarns require a protective coating to improve the yarn strength and to reduce the yarn hairiness (Livengood and Tomasino, 1980). Starch or size is the material that helps weavers ensure that their warp yarns are not broken during weaving. Otherwise, there would be a high machine breakdown rate, which would lead to low productivity. Sizing agents are selected on the basis of fabric type, environmental friendliness, ease of removal, cost considerations, effluent treatment, and others. As the starch or size covers the warp yarns and ultimately the fabric, it will lead to uneven dyeing, printing, or finishing. (Note: Warp yarn is only sized prior to the weaving process. In the knitting process, starch or size is not applied to the warp yarns. Therefore, there is no need to desize knitted material.)

Starch and its derivatives are the sizes most widely used for coating of warp yarns to prevent the threads from breaking during weaving. Starch is a cheap, natural raw material and it is biodegradable. Starch is a mixture of two polysaccharides, amylose and amylopectin, consisting mainly of α -(1,4)-linked glucose units. Carboxymethyl cellulose (CMC) and poly(vinyl alcohol) (PVA) are frequently used sizing agents for cotton yarns (Cacho, 1980; Moreland, 1980). A major negative aspect of PVA in size formulations is the difficulty of removing the PVA during desizing. After weaving, the size must be removed, since sized fabrics are less absorbent, especially for dyes. Desizing can be carried out by prolonged cooking or by using strong chemicals, such as acids, bases, or oxidizing agents.

The main objectives of desizing process are:

1. To remove the size material from the warp yarns in woven fabrics.
2. To ensure the levelness of the dyestuff or printing ink applied during dyeing or printing. If the size is not taken out completely, it will also affect the washing and rubbing fastness of the fabrics.

Desizing, irrespective of the desizing agent, involves impregnation of the fabric with the agent, allowing the agent to degrade or solubilize the size material, and finally to wash out the degradation products.

The major desizing processes are:

- Enzymatic desizing of starches on cotton fabrics
- Oxidative desizing
- Acid desizing
- Removal of water-soluble sizes: rot steeping

Of these methods, enzymatic desizing is the most widely practiced to desize starch (Hardin, 2011). Enzymes are named after the compound they break down; for example, *amylase* breaks down amylose and amylopectin, *maltase* breaks down maltose, and *cellulase* breaks down cellulose.

Originally, malt extract was used for the removal of amylaceous sizes from the fabric. Around 1900, diastafor was found more efficient for starch desizing. Rapidases, introduced in 1919, aid the liquefaction of starch into compounds in water. At present, commercial products based on amylopectic enzymes are widely used by the industry. These enzymes do not damage the cellulose of cotton fiber. They are also effective at various temperatures ranging from 20 to 115°C, covering all means of application. Nowadays, special attention is paid to the development of simultaneous desizing and scouring in an alkaline medium, replacing the two-stage process.

For desizing starch, amylase and maltase are generally used on a commercial scale. Enzymatic treatment with amylase enzymes has replaced the harsh processes used since the beginning of the twentieth century. Amylase removes starch “size.” It specifically hydrolyzes starch but does not affect cellulose. The product range of amylases includes mesophilic and thermophilic enzymes, allowing desizing at 20 to 115°C. Several applications can be used for desizing, such as padding, jigger, and continuous high-speed processes, in which the reaction time for the enzyme may be as little as 15 s. However, there is still considerable scope for improving the speed, economics, and consistency of the process, including the development of more heat-stable enzymes, optimization of biological oxygen demand levels, and a better understanding of how to characterize their activity and performance with respect to various fabrics, sizes, and processing conditions. Many commercial α -amylases are available, and it is estimated that approximately 15% of all commercial textile enzymes are used in desizing processes.

There are three types of amylases: bacterial, malt, and pancreatic. These enzymes are the starch-degrading agent of the fermentation process that occurs when starched fabric stored under wet conditions at slightly elevated temperatures becomes infected by microorganisms. The activity of the enzymes is affected by the presence of salts, as noted in Table 11.2.

Pancreatic enzyme is completely ineffective in the absence of salt. For full activity, sodium chloride (1.7 g/L) must be present. The presence of calcium chloride also activates the enzyme. Malt and bacterial enzymes require the presence of calcium for full activity.

Most amylases are suitable for continuous (pad-steam) as well as discontinuous desizing (jigger, winch, and pad-batch). The average liquor pickup depends on the characteristics of fabric, such as porosity and the additives present in the bath. Chelating agents should preferably not be used during the desizing process because calcium ions (at ppm levels) stabilize the enzymes. Wetting agents and nonionic surfactants can be used to enhance enzyme penetration and adsorption as well as fiber swelling, and to promote the removal of waxes, soils, and synthetic sizing agents. Nonionic surfactants are most suitable for enzymatic desizing, as anionics and cationics inactivate the enzymes through denaturation.

TABLE 11.2. Summary of Operational Conditions for Amylases

Amylase	Optimum pH	Optimum Temp. (°C)	Effect of NaCl	Effect of CaCl ₂
Pancreas	6.8–7.0	40–55	+	+
Malt α	4.6–5.2	55–65	–	+
Malt β	4.6–6.2	40–50	–	–
Bacterial	5.0–7.0	60–70	0	+

Desizing on a jigger is a simple method whereby the fabric from one roll is processed in a bath and rewound on another roll. First, the sized fabric is washed in hot water at 80 to 95°C to gelatinize the starch. The desizing liquor is then adjusted to pH 5.5 to 7.5 and a temperature of 60 to 80°C, depending on the type of enzyme. The fabric then goes through an impregnation stage before the amylase is added. Degraded starch in the form of dextrins is then removed by washing at 90 to 95°C for 2 minutes.

The jigger process is a batch process. By contrast, in modern continuous high-speed processes, the reaction time for the enzyme may be as short as 15 s. Desizing on pad rolls is continuous in terms of the passage of the fabric. However, a holding time of 2 to 16 h at 20 to 60°C is required using low-temperature α -amylases before the size is removed in washing chambers. With high-temperature amylases, desizing reactions can be performed in steam chambers at 95 to 100°C or even higher temperatures to allow a fully continuous process.

The mechanism of enzymatic desizing is as follows:

- Wetting of the substrate and pH buffering
- Swelling, penetration, cracking, and destabilization of the size layers
- Enzyme attack, the enzyme playing the role of molecular scissors
- Wash-off, dispersion of the degradation products

The actual desizing process comprises three important steps: saturation of the cloth with enzyme, reaction of the enzyme, and washing of the degraded products. The cloth is passed through the enzyme solution in either rope or open width form and is then squeezed and piled in pits. The desizing solution generally contains 0.1% enzyme and 0.2% common salt based on the weight of the fabric. The pH and temperature of the solution and concentration of enzyme that are required are maintained in the bath. After 8 to 10 h, or preferably overnight, the cloth is washed with water on a washing machine to remove the degraded starch products. If prior singeing is required, after singeing the cloth is passed through the enzyme solution in a continuous manner.

Desizing with extremophilic biocatalyst (enzymes) has several advantages over other desizing processes:

- Fewer chemicals are involved.
- The process is ecofriendly.
- It is efficient over a wide temperature range.
- No damage occurs to the cellulose fibers.
- There is better strength retention.
- There is softer fabric feel.
- There is a shorter processing time.

11.4.3. Enzymes in Biopreparation of Textiles

At present, conventional preparation methods (i.e., scouring and bleaching of cotton fabrics) involve the use of high concentrations of alkali, hydrogen peroxide, and other chemicals, often combined with high temperatures, to ensure efficient removal of impurities prior

to dyeing. These wet processes can cause depolymerization of cellulose molecules and significant losses in fabric weight and tensile strength. Furthermore, large quantities of water and energy are used and the alkaline effluents require special treatment. It is found that about 75% of the organic pollutant level arising from textile finishing is derived from the preparation of cotton goods.

Investigators at several centers have shown that impurities in cotton, such as pectins, proteins, and waxes, can be removed by specific enzymes, such as pectinases, proteinases, cellulases, and lipases, to increase the wettability and increase the whiteness of the fabric. Enzymatic biopreparation of cotton represents a rather new approach and is still primarily in the developmental stage. A prototype continuous scouring machine has already been developed. Cellulases have been reported to be most efficient in improving the wettability of cotton, and cellulase treatment applied before alkaline scouring has been shown to improve the removal of seed-coat fragments from desized cotton. Enzymatic bleaching of cotton fabric with glucose oxidase, producing H_2O_2 , has also been performed successfully. A process has been established that allows enzymatic desizing, bioscouring, and enzymatic bleaching with glucose oxidase to be combined. This process has yielded products with acceptable whiteness very close to that of commercially bleached goods, in addition to excellent mechanical properties.

Scouring Process. Scouring is a process designed to remove natural fat, wax, and oil from cotton fabrics using sodium hydroxide and a good detergent at boil for 20 to 30 min. Synthetic fabrics and other protein fabrics may use only sodium carbonate (a weaker alkali) instead.

The main objectives of the scouring process are:

- To remove natural fat, wax, and oil contained in fabrics without damaging the fibers
- To accelerate dye and chemical absorption of fabrics
- To improve the handle of goods (to make them softer)

SCOURING CELLULOSIC FIBERS. Gray cotton contains both natural and added impurities in the form of fats, oils, waxy matter, and spin finishes, which reduce the absorbency of cotton. Before cotton yarn or fabric can be dyed, it goes through a number of processes in a textile mill. One important step is scouring: the complete or partial removal of the noncellulosic components of native cotton, such as waxes, pectins, hemicelluloses, and mineral salts, as well as impurities such as machinery and size lubricants. Scouring yields a fabric with high and even wettability that can be bleached and dyed successfully (Hartzell and Hsieh, 1998; Holme, 2004; Lu, 2005). Today, highly alkaline chemicals such as sodium hydroxide are used for scouring. These chemicals not only remove the impurities but also attack the cellulose, leading to a reduction in strength and a loss of weight of the fabric. Furthermore, the resulting wastewater has a high chemical oxygen demand, biological oxygen demand, and salt content.

Within the last decade, alternative and mutually related processes, called bioscouring and biopreparation, have been introduced which are based on enzymatic hydrolysis of pectin substrates in cotton. Enzymatic scouring of unscoured cotton fabric can be done using pectinase, cellulase, protease, lipase, and other enzymes (Rössner, 1993; Hartzell

and Hsieh, 1998; Buchert et al., 2000; Waddell, 2002). Cellulases and pectinases are very effective compared to proteases and lipases.

- Cellulases are especially well suited for scouring of cotton fabrics. The degree of whiteness of a cotton sample treated with cellulases is lower by only 8 to 10% to that of the degree of whiteness obtained using an alkaline boiled-off treatment (Hartzell and Hsieh, 1998).
- Pectinolytic enzymes can be used for enzymatic degradation of pectin adhering to cotton. Cotton fiber or their blends with other fibers can be treated with aqueous solutions containing protopectinases for 18 h at 40°C to give scoured yarns with good tensile strength retention.

Pectinases can destroy the cuticle structure by digesting the inner layer of pectins in the cuticle of cotton. Cellulases can destroy the cuticle, digesting the primary wall of cellulose immediately under the cuticle of cotton. By combining the enzyme treatment (simultaneous treatment of pectinase and cellulase) or alkaline boiled-off treatment with an alkaline peroxide bleaching, the total degree of whiteness is higher. Cellulases break the linkage from the cellulose side, and the pectinases break the linkage from the cuticle side. The result of the synergistic effect gives more effective scouring in both the speed and evenness of the treatment.

Scourzyme L is an alkaline pectinase used to bioscour natural cellulosic fibers such as cotton, flax, hemp, and blends. It removes pectin from the primary cell wall of cotton fibers without degradation of the cellulose, and thus has no negative effect on the strength properties of cotton textiles or yarn.

Scouring with enzymes has a number of potential advantages over traditional processes:

- Total water consumption is reduced by 25% or more.
- The treated yarn or fabric retains its strength properties, and the weight loss is much less than for processing in traditional ways.
- Softer cotton textiles can be produced.

Enzymatic biopreparation of cotton represents a rather new approach and is still primarily in the development stages. A prototype continuous scouring machine has already been developed (Takagishi et al., 2001).

Bleaching Process. This process is used to remove the yellowness of a substrate and to improve its whiteness. This will help the goods to absorb more dyes and chemicals and will make the dye on the goods appear brighter. Normally, for cotton fabrics, sodium hypochlorite at room temperature and hydrogen peroxide in an alkaline solution at boil are the most popular bleaching agents (Jenkins, 2003). The main objectives of the bleaching process are:

- To whiten goods
- To make goods suitable for dyeing and printing in pale or bright shades
- To be followed by the addition of an optical brightening agent (a superwhite process)

BLEACHING COTTON AND JUTE. The natural pigments present in raw cotton are responsible for the grayness of the substrate. These pigments are removed completely during bleaching. Bleaching can be carried out with various oxidizing agents, such as sodium or calcium hypochlorite, hydrogen peroxide, or sodium chlorite. Recently, enzymes have been employed to remove natural coloring matter from cotton material. In the Synbleach project, natural fibers are bleached with hydrogen peroxide followed by enzymes, photosensitizers, and ultraviolet light. Various methods of bleaching paper pulp using enzymes have also been reported in the literature.

Cellulase, xylanase, and pectinase enzymes have a tremendous effect on the processing of jute. The treatment of enzyme before bleaching improves whiteness. The enzyme action is even greater on 4% NaOH-scoured fabric. Scouring causes higher hemicellulose loss, producing an open structure, and thus a larger surface area of lignin is accessible to hydrogen peroxide, resulting in higher whiteness.

The use of catalase enzymes to break down residual hydrogen peroxide after bleaching cotton is an already well established application (Weible, 1991). The catalase enzyme decomposes 2 mol of hydrogen peroxide into 2 mol of water and 1 mol of oxygen. The high rate of enzymatic degradation of hydrogen peroxide allows the reduction of water consumption during washing of the bleached cotton and prevents problems in later dyeing (Jensen, 2000; Tzanov et al., 2001).

Enzymatic bleaching of cotton fabric with glucose oxidase, producing H_2O_2 , has been investigated by Buschle-Diller et al. (2001). They established a process allowing the combination of enzymatic desizing, bioscouring, and enzymatic bleaching with glucose oxidase. According to their results, the process yielded products with acceptable whiteness very close to that of commercially bleached goods, and excellent mechanical properties.

11.5. FINISHING WITH EXTREMOPHILIC ENZYMES

Finishing is the last operation in the wet processing of textiles. In the processing of textiles, a variety of chemicals are used at different stages to enhance the quality of the substrate. These chemicals may be in the form of chlorinated scouring and bleaching agents, banned amine-based dyestuffs, stain removers, formaldehyde-based dye fixatives, cross-linking agents, softeners, and preservatives, among others. These substances are usually toxic and hazardous in nature. They cause tremendous damage not only to the environment but are also hazardous to the health of the person who uses the product. Conventional chemical processes are generally severe and may cause deterioration of the fiber structure. However, a high-quality surface appearance can also be given to visually unsightly and uneven fabrics through biocatalytic modification, commonly known as biofinishing. This is virtually a treatment with extremophilic enzymes to improve attractiveness, smoothness, and serviceability or to impart certain desirable properties to a fabric. An enzymatic treatment is usually carried out on cotton substrates for improving softness and smoothness of the material and also in denim washing.

11.5.1. Biopolishing of Cotton

The process of biopolishing employs the application of cellulase enzymes on cotton fabrics to prevent pilling and to impart smoothness and softness. In a conventional process, the

protruding fibers on the surface are removed by singeing, and softness is obtained through a chemical treatment. However, these conventional methods are temporary. After a few washes, the softening chemicals are removed during subsequent washing and laundering, while fibers rise on the surface of the material and fuzz is obtained. On the other hand, biopolishing offers the following advantages:

- Improved pilling resistance
- A clearer lint- and fuzz-free surface structure
- Improved drapeability and softness
- Durability

Biopolishing can be used to clean up a fabric surface after the primary fibrillation of a peach skin treatment and prior to a secondary fibrillation process, which imparts interesting fabric aesthetics (Hemmpel, 1991; Verma and Nishkam, 2002; Muthu and Ganesh, 2004). The biopolishing process requires:

- Enzyme dosage: 1 to 5% based on fabric weight (depending on the activity of the enzyme)
- Liquor ratio: 5 to 15 L/kg fabric
- Time: 60 to 120 min (depending on the amount of hydrolysis required)
- Temperature: 50 to 60°C
- pH: 4.5 to 5.5 (for acid-stable cellulose)

A weight loss in the base fabric of 3 to 5% is typical, but reduction in fabric strength can be controlled to within 2.7% by terminating the treatment after about 30 to 40 min using high temperature or low pH. Biopolishing can be carried out by both continuous and batch processes, but the treatment conditions are more easy to control in a batch-type process. Commercial products for biopolishing may contain only one or more cellulase enzymes in combination with each other. The monocomponent-based products contain only one cellulase enzyme, typically an endoglucanase, to provide a special effect to the substrate, whereas multicomponent-based products are designed to hydrolyze cotton to glucose, and hence they contain a range of different cellulases, four endoglucanases, two exocellulases, and a cellobiase.

11.5.2. Bio-Denim Washing

Indigo-dyed denim jeans are by far the most popular garment-processed items; approximately 1 billion pairs of jeans are produced annually. Traditionally, denim has been woven with cotton in a warp face twill weave in which the warp is blue and the weft is white. Dyeing is generally performed with pure indigo, or indigo combined with a sulfur dye to decrease the cost caused by the more expensive indigo dye. Indigo dye is popular because it washes down to clear bright blue shades without staining the white weft yarns.

Most denim jeans or other denim garments are subjected to a wash treatment to give them a slightly worn look. In the traditional stonewashing process, the blue denim is faded by the abrasive action of lightweight pumice stones on the garment surface, which removes



Figure 11.4. Enzyme-faded jeans. (See insert for color representation of the figure.)

some of the dye (Fig. 11.4). However, too much abrasion can damage the fabric, particularly hems and waistbands. This is why denim finishers today use cellulases to accelerate the abrasion by loosening the indigo dye on the denim. Since a small dose of enzyme can replace several kilograms of stones, the use of fewer stones results in less damage to garments, less wear on machines, and less pumice dust in the working environment. The need for the removal of dust and small stones from the finished garment is also reduced. Productivity can be increased further through laundry machines containing fewer stones and more garments. There is also no sediment in the wastewater, which can otherwise block drains.

The mode of action of cellulases can be explained as follows. Denim garments are dyed with indigo, a dye that penetrates only the surface of the yarn, leaving the center light in color. The cellulase molecule binds to an exposed fibril (bundles of fibrils make up a fiber) on the surface of the yarn and hydrolyzes it, leaving the interior part of the cotton fiber intact. When the cellulases partly hydrolyze the surface of the fiber, the blue indigo is released, aided by mechanical action, from the surface and light areas become visible, as desired.

Both neutral cellulases acting at pH 6 to 8 and acid cellulases acting at pH 4 to 6 are used for the abrasion of denim. A number of cellulases are available, each with its own special properties. These can be used either alone or in combination in order to obtain a specific look. Practical, ready-to-use formulations containing enzymes are available.

Laccase is an enzyme that is able to decolorize indigo and is sometimes used for the bleaching of denim garments. Since laccase alone is not capable of breaking indigo structure, it is used in combination with a mediator. In an aqueous medium, the enzyme gets

oxidized, which attacks the mediator to convert it to a free radical. The free radicals attack indigo and convert it into oxidized products. This enzymatic system has made it possible to get rid of hazardous chlorine-based bleaching agents (Kathiervelu, 2002).

When indigo is released to the wash liquor during washing, the solution turns dark blue. Indigo dye has two amino groups, which are capable of getting protonated in acidic media. Due to protonation, the dyestuff gains an overall positive charge, while cellulose maintains its negative charge in the acidic medium. The positive and negative charges attract one another in solution. This results in a decrease of the pH of the bath, and thereby the affinity of indigo for cotton increases. Some of the indigo redeposits on the white parts of the denim fabric, giving a lighter blue shade again and a dull look to the material. This phenomenon of the redeposition of released indigo onto the garments is known as back-staining (Schmitt and Prasad, 1998). This effect is very important in denim finishing. Application research in this area is focused on preventing or enhancing back-staining, depending on the style required. Back-staining at low pH values (pH 4 to 6) is relatively high, whereas it is significantly lower in the neutral pH range. Neutral cellulases are therefore often used when the objective is minimal back-staining.

The denim industry is driven by fashion trends. The various cellulases available (such as the DeniMax product range) for modifying the surface of denim give fashion designers a pallet of possibilities for creating new shades and finishes. Bleaching or fading of the blue indigo color can also be obtained by use of another enzyme product, DeniLite, which is based on a laccase and a mediator compound. This system, together with oxygen from the air, oxidizes and thereby bleaches indigo, creating a faded look. This bleaching effect was previously only obtainable using harsh chlorine-based bleach. The combination of new looks, lower costs, shorter treatment times, and less solid waste has made abrasion and bleaching with enzymes the most widely used fading process today. Incidentally, since the denim fabric is always sized, the complete process also includes desizing of the denim garments by the use of amylases.

Novo Nordisk (currently Novozymes) has also patented a method in which a stonewashing effect of denim was obtained by using xyloglucan polymer prior to dyeing and afterward creating an abraded or worn look by enzymatic degradation of the polymer using xyloglucanase. The xyloglucanase is not able to hydrolyze the cellulosic fabric, and therefore no strength loss is resulted.

11.5.3. Biopolishing of Jute

Bast fibers such as flax, jute, and ramie consist of a large amount of cellulose microfibrils embedded in a continuous phase, of which hemicellulose and lignin are predominant. Cellulases are generally used to remove the protruding fibers from the surface and modification of the surface structure of the jute fiber, thereby making it soft and smooth. The effect is more pronounced with the use of pectinases and xylanases. Commercial cellulase alone extensively removes protruding fibers from jute fabrics, whereas additions of pectinases or xylanases mainly loosen the protruding long jute fiber bundle and make their removal easier.

“Facing up” is the trade term for the ruffling up of the surface of wool garments by abrasive action during dyeing. Enzymatic treatment with proteases reduces facing up,

which significantly improves the pilling performance of garments and increases softness (Shreenath et al., 1996).

11.5.4. Biopolishing of Wool

Wool is a protein fiber composed of different amino acids joined by a peptide linkage. Proteases are employed commercially for wool biopolishing. These enzymes specifically act on the peptide bond of proteins to hydrolyze them. The part of the protease where cleavage action takes place, termed an active site, has a three-dimensional structure that determines the specificity of the protease. Different degrees of specificity exist, ranging from almost no specific proteases to highly specific proteases. Complete hydrolysis of wool, yielding free amino acids, is possible with such enzymes (Smith et al., 1995).

11.5.5. Biopolishing of Silk

Silk is the only natural fiber available in continuous filament form. Its main constituents are fibroin and sericin (a proteinaceous substance that covers the silk fiber). Generally, sericin has to be removed from the fiber by a degumming process to obtain the real luster for which the fiber is famous. Traditionally, degumming is performed in an alkaline solution containing soap. This is a harsh treatment because the fiber itself, the fibrin, is also attacked. However, the use of selected proteolytic enzymes is a better method because they remove the sericin without attacking the fibrin. Tests with high concentrations of enzymes show that there is no fiber damage and the silk threads are stronger than with traditional treatments. Scanning electron micrographs of the enzyme-treated samples show complete removal of the deposits on the surface of the fabric, which results in the biopolishing of the fabric, as indicated by the smooth clean surface of the fabric in the micrographs. Thus, the treatment of silk with a proteolytic enzyme results in completely clean fabric with a better biopolished finish. The cleaning of the surface of the enzyme is responsible for the improvement in the luster of silk (Gulrajani and Gupta, 1994).

11.5.6. Biopolishing of Lyocell

For cotton fabrics, the use of biopolishing is optional for upgrading the fabric. However, biopolishing is almost essential for the new type of regenerated cellulosic fiber *lyocell* (the leading make is known by the trade name Tencel). Lyocell is made from wood pulp and is characterized by a high tendency to fibrillate when wet. In simple terms, fibrils on the surface of the fiber peel up. If they are not removed, finished garments made of lyocell will end up with an unacceptable pilled look. This is the reason that lyocell fabric is treated with cellulases during finishing. Cellulases also enhance the attractive silky appearance of lyocell (Novozymes report, 2006).

Commercial application of cellulase enzymes requires prefibrillation in which the surface is fibrillated to the maximum extent. The fabric or garments are then subjected to enzymatic defibrillation using a cellulase enzyme, which weakens the ends of the fibers protruding from the fabric, while mechanical stress breaks off the weakened fibers, making the surface clean (Kumar et al., 1996; Kumar and Harnden, 1999).

11.6. ROLE OF ENZYMES IN TEXTILE AFTER-CARE

Dirt comes in many forms and includes proteins, starches, and lipids. In addition, clothes that have been starched must be freed of the starch. Using detergents in water at high temperatures and with vigorous mixing, it is possible to remove most types of dirt, but the cost of heating the water is high and lengthy mixing or beating will shorten the life of clothing and other materials. The use of enzymes allows lower temperatures to be employed, and shorter periods of agitation are needed, often after a preliminary period of soaking. In general, enzyme detergents remove protein from clothes soiled with blood, milk, sweat, or grass far more effectively than do nonenzyme detergents. The use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents presently available containing enzymes. Enzymes have contributed greatly to the development and improvement of modern household and industrial detergents, the largest application area for enzymes today. They are effective at the moderate temperature and pH values that characterize modern laundering conditions. In laundering and in industrial and institutional cleaning, they contribute to:

- Better cleaning performance in general
- Rejuvenation of cotton fabric through the action of cellulases on fibers
- Reduced energy consumption by enabling lower washing temperatures
- Reduced water consumption through more effective soil release
- Minimal environmental impact, since they are readily biodegradable
- Environment-friendly wash-water effluents (in particular, phosphate-free and less alkaline)

Furthermore, the fact that enzymes are renewable resources also makes them attractive to use from an environmental point of view.

Enzyme applications in detergents began in the early 1930s with the use of pancreatic enzymes in presoak solutions. The German scientist Otto Rohm patented the first use of pancreatic enzymes in 1913. The enzymes were extracted from the pancreases of slaughtered animals and included proteases (trypsin and chymotrypsin), carboxypeptidases, α -amylases, lactases, sucrases, maltases, and lipases. Thus, with the exception of cellulases, the foundation was laid in 1913 for the commercial use of enzymes in detergents. Today, enzymes are growing in importance continuously for detergent formulators. The most widely used detergent enzymes are hydrolases, which remove soils formed from proteins, lipids, and polysaccharides.

Looking to the future, research is currently being carried out into the possibility of extending the types of enzymes used in detergents. Each of the major classes of detergent enzymes—proteases, lipases, amylases, mannanases, and cellulases—provides specific benefits for laundering. Historically, proteases were the first to be used extensively in laundering. Today, they have been joined by lipases, amylases, and mannanases in increasing the effectiveness of detergents, especially for household laundering at lower temperatures and, in industrial cleaning operations, at lower pH. Cellulases contribute to cleaning and overall fabric care by rejuvenating or maintaining the appearance of washed cotton-based garments. The detailed characteristics of the detergent extremophilic enzymes utilized in

“Biological” washing powders are as follows (Cavaco-Paulo et al., 1996a,b; Shirish Kumar, 2007):

- *Proteases*. Proteases are the most widely used enzymes in the detergent industry. They remove protein stains such as grass, blood, milk, tomato sauce, egg, and human sweat. These organic stains have a tendency to adhere strongly to textile fibers. The proteins act as glues, preventing the waterborne detergent systems from removing some of the other components of the soiling, such as pigments and street dirt. The inefficiency of nonenzymatic detergents at removing proteins can result in permanent stains due to oxidation and denaturing caused by bleaching and drying. Blood, for example, will leave a rust-colored spot unless it is removed before bleaching. Proteases hydrolyze proteins and break them down into more soluble polypeptides or free amino acids. As a result of the combined effect of surfactants and enzymes, stubborn stains can be removed from fibers.
- *Lipases*. Although enzymes can easily digest protein stains, oily and fatty stains have always been troublesome to remove. The trend toward lower washing temperatures has made the removal of grease spots an even bigger problem. This applies particularly to materials made up of a blend of cotton and polyester. The lipase is capable of removing fatty stains such as fats, butter, salad oil, sauces, lipsticks, and the tough stains on collars and cuffs.
- *Amylases*. Amylases are used to remove residues of starch-based foods such as potatoes, spaghetti, custards, gravies, rice, pasta, and chocolate. This type of enzyme can be used in laundry detergents as well as in dishwashing detergents.
- *Cellulases*. The development of detergent enzymes has focused on enzymes capable of removing stains. However, a cellulase enzyme has properties enabling it to modify the structure of cellulose fiber on cotton and cotton blends. Cellulase is a type of hydrolase that provides fabric care through selective reactions not previously possible when washing clothes. When it is added to a detergent, it helps to keep cotton look new by brightening the colors of the garments, maintaining soft feel, and assisting in soil removal in the following manner:
 - *Color brightening*. When garments made of cotton or cotton blends have been washed several times, they tend to get a “fluffy” look and the colors become duller. This effect is due to the formation of microfibrils that become partly detached from the main fibers. The light falling on the garment is reflected back to a greater extent, giving the impression that the color is duller. These fibrils, however, can be degraded by the cellulase enzyme, restoring a smooth surface to the fiber and restoring the garment to its original color.
 - *Softening*. The enzyme also has a significant softening effect on the fabric, probably due to the removal of the microfibrils.
 - *Soil removal*. Some dirt particles are trapped in the network of microfibrils and are released when the microfibrils are removed by the cellulase enzyme.

Enzymes are used in surprisingly small amounts in most detergent preparations, only 0.4 to 0.8 wt% crude enzyme (about 1% by cost). Once released from its granulated form, the enzyme must withstand anionic and nonionic detergents; soaps; oxidants such as sodium

perborate, which generate hydrogen peroxide; optical brighteners; and various less reactive materials, all at pH values between 8.0 and 10.5.

The enzymes used are all produced using species of *Bacillus*, mainly by just two companies:

- Novo Industri A/S produces and supplies three proteases: alcalase, from *B. licheniformis*; esperase, from an alkalophilic strain of a *B. licheniformis*; and savinase, from an alkalophilic strain of *B. amyloliquefaciens* (often mistakenly attributed to *B. subtilis*).
- GistBrocades produces and supplies maxatase, from *B. licheniformis*. Alcalase and maxatase (both mainly subtilisin) are recommended for use at 10 to 65°C and pH 7 to 10.5. Savinase and esperase may be used at up to pH 11 and 12, respectively. The α -amylase supplied for detergent use is termamyl, the enzyme from *B. licheniformis*, which is also used in the production of glucose syrups. α -Amylase is particularly useful in destarching detergents.

In addition to the granulated forms intended for use in detergent powders, liquid preparations in solution in water and slurries of the enzyme in a nonionic surfactant are available for formulating in liquid spotting concentrates, used for removing stubborn stains. Preparations containing both termamyl and alcalase are produced, termamyl being sufficiently resistant to proteolysis to retain activity for long enough to fulfill its function.

The enzymes are supplied in forms (as described above) suitable for formulation by detergent manufacturers. Domestic users are familiar with powdered preparations, but liquid preparations for home use are increasingly available. Household laundering presents problems quite different from those of industrial laundering: The household wash consists of a great variety of fabrics soiled with a range of materials, and the user requires convenience and effectiveness with less consideration of the cost. Home detergents will probably include both an amylase and a protease, and a lengthy warm-water soaking time will be recommended. Industrial laundering requires effectiveness at minimum cost, so heated water will be reused if possible. Large laundries can separate their "wash" into categories and thus minimize the use of water and maximize the effectiveness of the detergents. Thus, white cotton uniforms from an abattoir can be segregated for washing, only protease being required. A prewash soaking for 10 to 20 min at pH up to 11 and 30 to 40°C is followed by a main wash for 10 to 20 min at pH 11 and 60 to 65°C. The water from these stages is discarded to the sewer. A third wash includes hypochlorite as bleach, which would inactivate the enzymes rapidly. The water from this stage is used again for the prewash, but by then the hypochlorite concentration is insufficient to harm the enzyme.

This is essentially a batch process; hospital laundries may employ continuous washing machines, which transfer less-initially-dirty linen from a prerinse initial stage at 32°C and pH 8.5 into the first wash at 60°C and pH 11, then to a second wash, containing hydrogen peroxide, at 71°C and pH 11, then to a bleaching stage and rinsing. Apart from the presoak stage, from which water is run to waste, the process operates countercurrently. Enzymes are used in the prewash and in the first wash, the levels of peroxide at this stage being insufficient to inactivate the enzymes (Galante and Formantici, 2003).

There are opportunities to extend the use of enzymes in detergents both geographically and numerically. They have not found widespread use in developing countries, which are

often hot and dusty, making frequent washing of clothes necessary. The recent availability of a suitable lipase may increase the quantities of enzymes employed very significantly. There are, perhaps, opportunities for enzymes such as glucose oxidase, lipoxygenase, and glycerol oxidase as means of generating hydrogen peroxide in situ. Added peroxidases may aid the bleaching efficacy of this peroxide.

A recent development in detergent enzymes has been the introduction of an alkaline-stable fungal cellulase preparation for use in washing cotton fabrics. During use, small fibers are raised from the surface of cotton thread, resulting in a change in the “feel” of the fabric and, particularly, in the lowering of the brightness of colors. Treatment with cellulase removes the small fibers without apparently damaging the major fibers and restores the fabric to its “as new” condition. The cellulase also aids the removal of soil particles from the wash by hydrolyzing associated cellulose fibers.

The obvious advantages of enzymes make them universally acceptable for meeting consumer demands. Due to their catalytic nature, they are ingredients requiring only a small space in the formulation of the overall product.

11.7. ROLE OF ENZYMES IN EFFLUENT TREATMENT OF TEXTILES

Natural and enhanced microbial processes have been used to treat waste materials and effluent streams from the textile industry. Conventional activated sludge and other systems are generally well able to meet biological oxygen demand and related discharge limits on most cases. The industry faces some specific problems such as color removal from dyestuff effluent and handling of toxic wastes, including pentachlorophenols (PCPs) and heavy metals (Willmott et al., 1998; Abadulla et al., 2000; Nyanhongo et al., 2002; Cing et al., 2003).

The synthetic dyes are designed such that they become resistant to microbial degradation under aerobic conditions. Also, the water solubility and the high molecular mass inhibit permeation through biological cell membranes.

Anaerobic processes convert the organic contaminants principally into methane and carbon dioxide and usually occupy less space, treat wastes containing up to 30,000 mg/L of chemical oxygen demand, have lower running costs, and produce less sludge.

Environmentally friendly pretreatment processes for textiles are the leading need of the day, due to tremendous awareness of chemical pollution and mounting legislation by governments to limit the chemical burden of factory effluent. Biotechnology can be used for the treatment of wastes, which can solve the problem either partially or totally. The application of biotechnology is mainly attributed to the removal of color from the dyehouse effluents. Living organisms are used to bind and degrade color (e.g., artificial reed beds) or dead organisms (e.g., straw, chitin/chitosan, microfungal hyphae). Selected microbes or isolated enzymes may be used to assist specific areas. The discharge of dyestuffs into the environment is not solely an aesthetic matter, and many dyestuffs are identified as mutagenic (Metosh-Dickey et al., 1999).

Biosorption has the potential to remove metal ions such as chromium, which are used in the manufacture and application of mordant dyes. The enzymatic decoloration of the dye released reduces process time and the amount of energy and water needed to achieve a satisfactory textile quality. However, the type and concentration of dyes as well as the amount and type of substrate used are found to play a major role in dye adsorption. Chitosan is found to be most efficient in adsorbing dyes of small molecular size.

Azo dyes are susceptible to anaerobic biodegradation, but reduction of azo compounds can result in odor problems. Biological systems such as biofilters and bioscrubbers are now available for the removal of odor and other volatile compounds. The dyes can be removed by biosorption on apple pomace and wheat straw. The experimental results showed that 1 g of apple pomace and 1 g of wheat straw, with a particle size of 600 μm , were suitable adsorbents for the removal of dyes from effluents. Apple pomace had a greater capacity than wheat straw to adsorb the reactive dyes taken for the study (Robinson et al., 2002).

11.8. CONCLUSIONS

Biotechnology finds wide application in textiles, and it will prove to be a boon to the ever-changing conditions of the ecology as well as the economy. The advent of biotechnological applications in textile processing widens the already existing wide horizons to produce aesthetically colorful magnanimous and ecologically friendly textiles, opening a new era of synergetic application of life sciences. These biotechnological applications, with the utilization of enzymophilic enzymes, are ecofriendly and not harmful to either the food web or the life cycle of any other living creature. Such awareness is gradually metamorphosing a tool that could be used intelligently to meet the demand of our fashion trends.

Enzymes, bacteria, and insects could be modified biologically into a fashion promoter if engineered with great caution. Textile processes based on enzymes have gained importance in view of the stringent environmental and industrial safety conditions. Amylases have long been used for cotton desizing. Cellulases are being used successfully in denim stonewashing. Enzymes show great promise in terms of effectiveness and applications. A major breakthrough in the textile industry is eagerly awaited through these biotechnological applications. Since enzymes can easily be deactivated and are biodegradable, they will be of great help to humankind in reducing environmental pollution in coming years. Furthermore, there are opportunities to combine enzyme technology with better process control to facilitate the production of value-added consumer goods.

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THE USE OF EXTREMOPHILIC MICROORGANISMS IN THE INDUSTRIAL RECOVERY OF METALS

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12.1. INTRODUCTION

The bioleaching of ores, or *biomining*, is the industrial microbial solubilization of ores to extract metals such as copper, gold, uranium, and others (Rawlings, 2005; Watling, 2006; Watling et al., 2010; Jerez, 2011). This process is done using extremophilic chemolithoautotrophic microorganisms that live in extremely acidic conditions (pH 1 to 3.0) and in the presence of very high toxic heavy metal concentrations. When acidophiles oxidize elemental sulfur and reduced sulfur species such as sulfides, sulfites, or polysulfides, they generate sulfuric acid, which acidifies their environment to pH values between 1 and 2 (Suzuki, 1999; Rohwerder et al., 2003). As a consequence, these microorganisms may also have an important impact on the environment, generating serious contamination with acid and toxic metals that can get into water sources as acid mine drainage (AMD) (Schippers, 2007; Jerez, 2009; Mohapatra et al., 2011).

A great variety of microorganisms are present in biomining commercial operations and at AMD sites. Figure 12.1 illustrates hypothetical behavior and the environmental adaptations with which a biomining community of microorganisms is confronted. The detailed molecular knowledge of the physiology of these extremophiles has greatly advanced in recent years, and it is expected that application of these new findings will improve biomining operations (Cárdenas et al., 2010; Watling et al., 2010; Jerez, 2011).

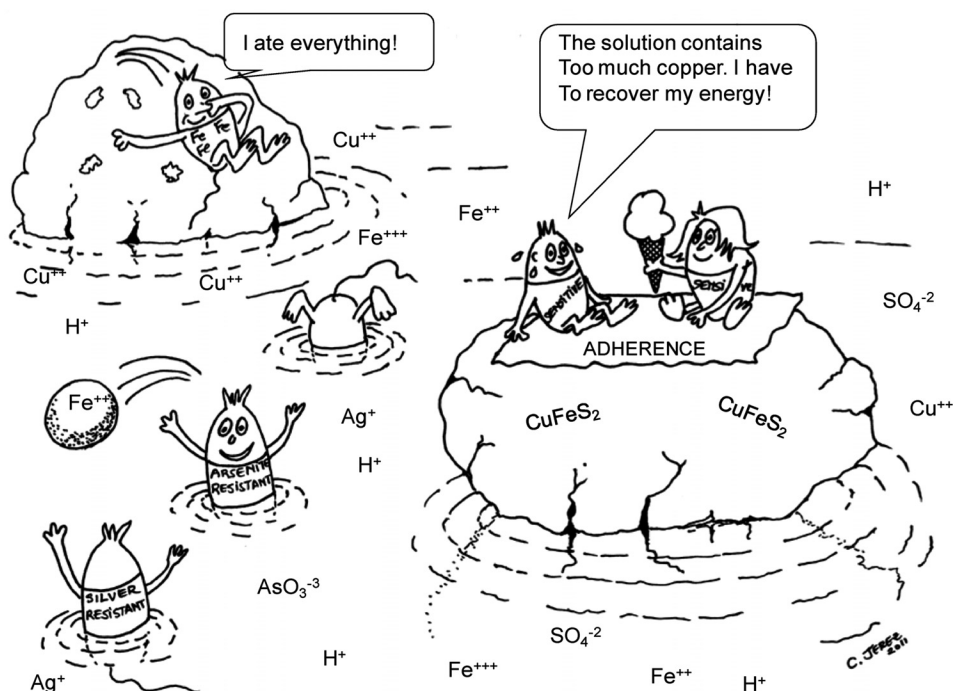


Figure 12.1. A cartoon reflecting the author's view of a possible way of life for a biomining microbial community.

12.2. BIOMINING EXTREMOPHILES AND THEIR INDUSTRIAL APPLICATIONS

Industrial biomining operations are of several types, depending on ore type and geographical location, the metal content, and the specific minerals present (e.g., metal oxides, metal sulfides of different kinds). One setup widely used for the recovery of gold and copper is irrigation. Such processes involve the percolation of leaching solutions through the crushed ore that can be contained in a column, a heap, or a dump (Watling, 2006; Jerez, 2011). In Figure 12.2 we see a scheme in which the crushed ore to be bioleached is transported to an agglomeration tank or drum, where it is acidified. This is a key process, since the larger ore particles are surrounded by very fine particles that stick to them, thus preventing all the particles especially the fine material sediment, from reaching the bottom of the heap. In this way, irrigation and aeration of the heap takes place from top to bottom, allowing a much more homogeneous growth of the microbial community and therefore better metal solubilization.

Heaps can vary in size but are usually up to 10 m tall and 100 or more meters long and wide and are constructed over irrigation pads lined with high-density polyethylene to avoid losses of the pregnant copper-containing solution. This solution contains copper sulfate generated by the microbial solubilization of the insoluble copper sulfides present in the ore. After solvent extraction of the pregnant solution, a highly concentrated copper sulfate solution is obtained. Finally, the metal is recovered in an electrowinning plant to

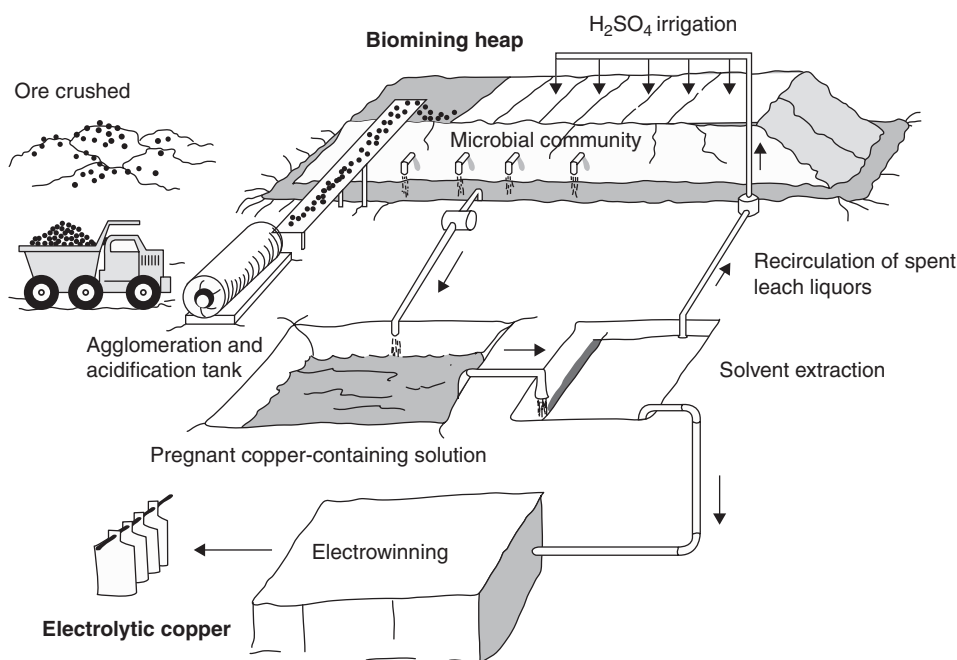


Figure 12.2. Typical heap bioleaching industrial operation to generate electrolytic copper.

generate electrolytic copper of high purity (Fig. 12.2). Since most mining operations are located in areas where water is scarce, the spent leach liquors or raffinates are usually recirculated to the heap for further irrigation.

Bioleaching bacteria can also be used for gold recovery (Rawlings, 2005). Gold is usually found in nature associated with minerals containing arsenic and pyrites (arsenopyrites). During gold bioleaching, the iron- and sulfur-oxidizing microorganisms attack and solubilize the arsenopyrite, releasing the trapped gold particles. Following this release, the gold is complexed with cyanide according to standard gold-mining procedures. Instead of using big leaching heaps or dumps as in the case of bioleaching of copper ores, gold bioleaching is usually done using highly aerated stirred-tank bioreactors connected in series. Since these reactors are expensive to build, they are used for high-grade ores or with mineral concentrates (Watling et al., 2010; Jerez, 2011). The advantage of tank reactors over heaps and dumps, which are “open bioreactors” is that conditions in the tanks can be controlled, leading to a much faster and efficient metal extraction process.

Currently, there are operations using both mesophilic and thermophilic microorganisms (Watling, 2006). Biomining does not require the large amounts of energy used during roasting and smelting in traditional mining processes, and it does not generate toxic gaseous emissions such as sulfur dioxide. Nevertheless, as already mentioned, AMD can be generated, which should be controlled properly to avoid environmental pollution. Biomining is also a great advantage since not only discarded low-grade ores from standard mining procedures, but also some high-grade ores, can be leached in an economically feasible way. In a country such as Chile, which is actually the first world copper producer, many mining operations process from 10,000 to 40,000 tons of ore per day and produce between

10,000 and 200,000 tons of copper per year by using heap or dump bioleaching of minerals such as oxides, chalcocite, covellite, chalcopyrite, and others (Watling et al., 2010; Jerez, 2011). Similar situations take place in the United States, Australia, and other countries. The most successful have been those processing copper oxides and secondary copper sulfides. However, chalcopyrite is the most abundant copper sulfide in the world. Since it is the most difficult to solubilize by microorganisms, there is actually great interest in developing processes mainly using thermophilic biomining microorganisms (Watling, 2006; Watling et al., 2010).

The most studied leaching bacteria are from the genus *Acidithiobacillus*. *A. ferrooxidans* and *A. thiooxidans* are acidophilic mesophiles and together with the moderate thermophile *A. caldus*, they belong to the gram-negative γ -proteobacteria. *A. ferrooxidans* is a chemolithoautotrophic bacterium that obtains its energy from the oxidation of ferrous iron, elemental sulfur, or partially oxidized sulfur compounds (Rohwerder et al., 2003; Rawlings, 2005).

Members of the genus *Leptospirillum* are other important biomining bacteria that belong to a new bacterial division, Nitrospora. Some gram-positive bioleaching bacteria belonging to the genera *Acidimicrobium*, *Ferrimicrobium*, and *Sulfobacillus* have also been described (Schippers, 2007 and references therein). Biomining extremely thermophilic archaeons capable of oxidizing sulfur and iron(II) have been known for many years, and they are mainly from the genera *Sulfolobus*, *Acidianus*, *Metallosphaera*, and *Sulfurisphaera*. Some mesophilic iron(II)-oxidizing archaeons belonging to the Thermoplasmatales have also been isolated and described: *Ferroplasma acidiphilium* and *F. acidarmanus*. In fact, a consortium of different microorganisms is responsible for the oxidative reactions, resulting in the extraction of dissolved metal values from ores (Schippers, 2007; Watling et al., 2010; Mohapatra et al., 2011 and references therein).

12.3. MOLECULAR STUDIES IN ACIDOPHILIC BIOMINING MICROORGANISMS

Knowing bacteria–mineral interactions is a key aspect for bioleaching of ores. Most leaching bacteria grow attached to solid substrates such as elemental sulfur and metal sulfides. This attachment is mediated predominantly by extracellular polymeric substances (EPSs) present on the surface of the cells. Bacteria adhere to the surface of the mineral, carrying Fe(III) bound to its exopolysaccharides, and when the microorganism forms a biofilm, this metal would chemically oxidize the metal sulfide, generating in the case of pyrite, ferrous iron that is reoxidized to Fe(III) and thiosulfate, which can be further oxidized to sulfuric acid (Rohwerder et al., 2003). This close contact of the bacterium with the mineral makes the sulfide oxidation more efficient and specific.

Insoluble metal sulfides are oxidized to soluble metal sulfates by the chemical action of ferric iron, the main role of the microorganisms being reoxidation of the ferrous iron generated to obtain additional ferric iron. Considerable effort has been spent in recent years to understand the biochemistry of iron and sulfur compound oxidation (Suzuki, 1999; Rohwerder et al., 2003; Rawlings, 2005; Valenzuela et al., 2006), bacteria–mineral interactions (chemotaxis, quorum sensing, adhesion, biofilm formation) (Jerez, 2011 and references therein), and several adaptive responses that allow the microorganisms to survive

in a bioleaching environment (Das et al., 1998; Dopson et al., 2003; Alvarez and Jerez, 2004; Quatrini et al. 2006; Remonsellez et al., 2006; Chi et al., 2007; Jerez, 2008; Orell et al., 2010; Mykytczuk et al., 2011). All of this knowledge is considered central to an understanding of the biomining process.

The reactions involved in ferrous iron oxidation by *A. ferrooxidans* have been studied in detail. In the electron pathway from ferrous iron to oxygen the terminal electron acceptor is assumed to be a cytochrome oxidase anchored to the cytoplasmic membrane. The transfer of electrons would occur through several periplasmic carriers, including at least the blue copper protein rusticyanin and cytochrome *c*552. A high-molecular-mass *c*-type cytochrome, Cyc2, has been implicated as the initial electron acceptor in the respiratory pathway between ferrous iron and oxygen (Yarzabal et al., 2002; Rawlings, 2005). This pathway would be composed of at least the electron carriers Cyc2, rusticyanin, Cyc1(*c*552), and aa3 cytochrome oxidase.

The aerobic oxidation of elemental sulfur by *A. ferrooxidans* and other microorganisms is carried out by a sulfur dioxygenase (Suzuki, 1999; Rohwerder et al., 2003; Rawlings, 2005). Thiosulfate has been postulated as a key compound in the oxidation of the sulfur moiety of pyrite. Iron(III) ions are exclusively the oxidizing agents for the dissolution. Thiosulfate would, consequently, be degraded in a cyclic process to sulfate, with elemental sulfur being a side product (Rohwerder et al., 2003; Rawlings, 2005).

Effective molecular tools for the study of biomining microorganism genetics and physiology are still scarce (Liu et al., 2000, 2007; van Zyl et al., 2008). To achieve efficiency and reproducibility of these procedures is still a rather difficult task. *A. ferrooxidans* was the first biomining microorganism to have its genome sequenced entirely (<http://www.tigr.org>). In the last decade, the number of genomic sequences has increased greatly, and this will continue due mainly to the great advances in automatic high-throughput DNA sequencing methods that have greatly reduced both the time and costs needed to generate microbial genomic sequences.

Recently, Cárdenas et al. (2010) reviewed the current advances on finished and on-going genomic sequencing projects related to biomining microorganisms. The genomic information has been and will be very useful to researchers searching for genome-wide candidate genes for important metabolic pathways and several important physiological functions and predicting the functions of many new genes. The main focus of research has been the energetic metabolism that is directly responsible for bioleaching, such as sulfur and iron metabolism (Rawlings, 2005; Valenzuela et al., 2006). The transcriptomics (Quatrini, et al., 2006; Auernik et al., 2008; Cárdenas et al., 2010; Moreno-Paz et al., 2010) and high-throughput proteomics studies of the global regulatory responses that the biomining microorganisms use to adapt to their changing environment are just beginning to emerge (Valenzuela et al., 2006; Chi et al., 2007). About 30% of the genes and proteins coded in these genomes are without homologs in databases, indicating that they are probably characteristic of each microorganism and may have important roles yet to be assigned.

Metagenomics is the culture-independent genomic analysis of microbial communities. In conventional shotgun sequencing of microbial isolates, all the shotgun DNA fragments are derived from clones of the same genome. To analyze the genomes of an environmental microbial community, the ideal situation is to have a low-diversity environment. Such systems were found when analyzing the microbial communities inhabiting a biofilm developed at a site of extreme AMD production. Random shotgun sequencing of DNA from

this natural acidophilic biofilm was used by Tyson et al. (2004), who could reconstruct the nearly complete genomes of *Leptospirillum* group II and *Ferroplasma* type II and partially recover three other genomes. The extremely acidic conditions of the biofilm (pH about 0.5) and relatively restricted energy source combine to select for a small number of species, a situation ideal for testing these new culture-independent genomic approaches in the environment (Tyson et al., 2004).

Analysis of the gene complement for each organism revealed the metabolic pathways for carbon and nitrogen fixation and energy generation. For example, genes for biosynthesis of isoprenoid-based lipids and for a variety of proton efflux systems were identified, providing insights into survival strategies in the extreme acidic environment. However, this information will have to be confirmed by biochemical and physiological approaches. Clearly, the metagenomic approach for the study of microbial communities is a real advancement to fully understand how complex microbial communities function and how their component members interact within their niches.

A metaproteomic approach has been used to study the community in a natural AMD microbial biofilm (Ram et al., 2005; Lo et al., 2007; Denef et al., 2010). These authors were able to detect over 2000 proteins from the five most abundant species in the biofilm, including 48% of the proteins predicted to be from the dominant biofilm organism, *Leptospirillum* group II. The authors also determined that one abundant novel protein was a cytochrome that is central to iron oxidation and acid mine drainage formation in the natural biofilm (Ram et al., 2005). Lo et al. (2007) used community genomic data sets to identify, with strain specificity, proteins expressed from the dominant member of a genomically uncharacterized natural acidophilic biofilm. Proteomics revealed interpopulation genetic exchange, which may be crucial for the adaptation to specific ecological niches within the very acidic metal-rich environment studied. All this knowledge, together with that obtained in other bioleaching microorganisms, will allow future improvements in industrial bioleaching and bioremediation processes. More recently, three different libraries obtained from an AMD biofilm community allowed the reconstruction of 12 nearly complete bacterial and archaeal genomes (Denef et al., 2010).

Substantial metagenomic advances in the biomining communities could be expected in the near future, thanks to the advent of much more economical high-throughput DNA sequencing procedures and improved bioinformatics tools.

12.4. MICROBIAL RESISTANCE TO ACID AND METALS

12.4.1. Acidophilic Bacteria

Acidophilic microorganisms have several active mechanisms to use to withstand their extremely acidic environment (Dopson et al., 2003; Krulwich et al., 2011). In addition, some passive mechanisms of pH homeostasis have been proposed to support the active mechanisms. Some strategic changes in proton permeability of the membrane and cell surface changes have been thought to delay proton entry into or loss from the cytoplasm (Krulwich et al., 2011). Examples of these possible passive systems have been reported for *A. ferrooxidans*, the biomining bacterium most studied. This microorganism has an unusual *pI* profile which may not only address functional needs but could also provide a passive contribution to the active mechanisms for pH homeostasis. Surface proteins in

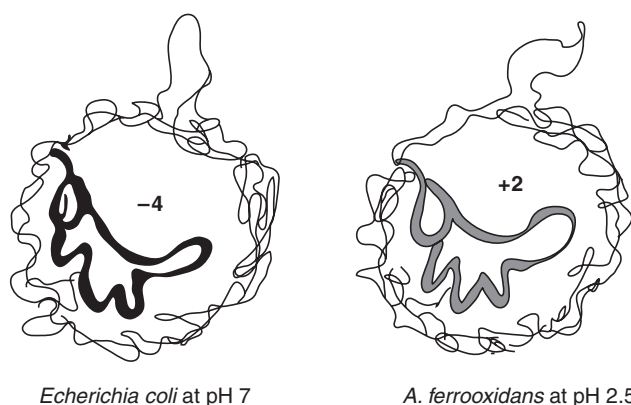


Figure 12.3. Passive mechanism of pH homeostasis in *A. ferrooxidans*; a cross section of a pore from an *E. coli* porin and OMP40, the major porin from *A. ferrooxidans*. The corresponding loop 3's, which fold inside the diffusion channel, are represented respectively in black or gray.

A. ferrooxidans have high *pI* values relative to neutrophilic bacteria (Chi et al., 2007), so their positive charges could act as a transient proton repellent at the cell surface. Adjustments of membrane lipids and porins have also been proposed in *A. ferrooxidans* to minimize inward proton leakage during acid stress (Krulwich et al., 2011 and references therein). Porins are organized in trimeric structures that form water-filled channels that allow diffusion of small molecules through the outer membrane. Each monomer constitutes a discrete pore. Within each pore of OmpC from *E. coli* a long polypeptide loop (L3) runs along one side of the barrel wall and narrows the pore to create an “eyelet” region (Nikaido, 1994). A cross section of a pore from *E. coli* is compared in Figure 12.3 with that possibly existing in OMP40, the major porin from *A. ferrooxidans* (Guiliani and Jerez, 2000).

The net electric charge of these loop 3's is important for the permeability of the molecules passing through the pore. The net charge of loop 3 in *A. ferrooxidans* would be +2 at its usual growth pH of 2.5. On the other hand, the equivalent loop in the neutrophilic *E. coli* would have a net charge of −4 at its growth pH of 7.0. Therefore, the *A. ferrooxidans* loop could act as a proton barrier to control the free entry of protons from the outside and thus avoid excessive acidification of their periplasmic space (Guiliani and Jerez, 2000).

As already mentioned, microorganisms that grow in mineral-rich environments are, in most cases, remarkably resistant to a wide range of metal ions (Dopson et al., 2003; Franke and Rensing, 2007; Orell et al., 2010). Therefore, microorganisms surviving in acid-leaching environments should possess strong metal resistance mechanisms. In this chapter I refer only to copper resistance. Although knowledge about resistance to other metals in biomining microorganisms is scarce, some recent reviews address this matter (Franke and Rensing, 2007; Auernik et al., 2008).

When cellular copper concentration exceeds acceptable levels, mechanisms of resistance are activated in order to survive this adverse environment (Rensing and Grass, 2003; Magnani and Solioz, 2007). In gram-negative bacteria, one of the pathways described for Cu resistance is the active efflux of metal from the cytoplasm to the periplasmic space carried out by ATPases located in the internal membrane of the bacteria (Rensing and Grass, 2003). Some microorganisms may pump metal from the cytoplasm directly to the

extracellular space by systems of the RND (resistance nodulation cell division) family of carriers, such as the Cus system of *Escherichia coli* (Outten et al., 2001). The capacity of some species to bind the metal in the periplasmic space by means of copper chaperones has also been reported (Puig et al., 2002).

Typically, concentrations of Cu in heap or dump leachates are in the range 30 to 90 mM. On the other hand, in agitated tanks, where sulfide concentrates are processed, the concentrations can reach up to about 300 mM or more (Watling et al., 2010). Metal tolerance can vary significantly between species and between strains of the same species (Orell et al., 2010; Watling et al., 2010). Given the differences between metal tolerance and metal concentrations in heap or agitated-tank bioleaching operations, it is important to further understand the mechanisms used by these microorganisms to adapt to and to resist the high concentrations of copper found in their environment.

In the process of bioleaching of a mineral such as chalcopyrite, iron and copper ions are produced. These two metals are known to generate reactive oxygen species (ROSs) in microorganisms through the Fenton reaction (Orell et al., 2010 and references therein). When grown in the presence of CuSO₄, several of the *A. ferrooxidans* putative oxidative stress genes, such as putative glutathione synthetase (*gshB*), glutathione reductase (*gor*), superoxide dismutase (*sod*), and alkylhydroperoxidase (*ahpC*), increased their expression levels compared to those in bacteria grown in a non-copper-containing mineral alone, such as pyrite (Orell et al., 2010). In addition, very recently, ROSs generated from mechanically activated sulfide concentrates that affect thermophilic bioleaching were described (Jones et al., 2011). It is therefore most likely that biomining microorganisms growing in Cu-containing minerals are subjected to oxidative stress. This stress response may be important to monitor during industrial bioleaching operations to assess the degree of toxicity generated by the metal ions and its effect on the efficiency of the bioleaching process.

In the environment of biomining microorganisms, copper concentrations are one or two orders of magnitude higher than those tolerated by neutrophiles. Thus, *A. ferrooxidans* can be adapted to grow in the presence of Cu concentrations higher than 300 mM, and the archaeon *Sulfolobus metallicus* is able to grow in 200 mM Cu (Dopson et al., 2003; Orell et al., 2010).

A. ferrooxidans ATCC 23270 can survive in high copper concentrations by having in its genome at least 10 genes that are possibly related to Cu homeostasis, among them three genes coding for putative ATPases related to the transport of Cu (*afcopA1*, *afcopA2*, and *afcopB*); three genes related to a system of the resistance nodulation cell division (RND) family, involved in the extraction of Cu from the cell (*afcusA*, *afcusB*, *afcusC*); and two genes coding for periplasmic chaperones for this metal (*afcusF* and *afcopC*) (Navarro et al., 2009; Wu et al., 2010). The putative *A. ferrooxidans* ATCC 23270 Cu-resistance determinants were found to be upregulated when this bacterium was exposed to Cu in the range 5 to 25 mM, and these genes conferred on *E. coli* a greater Cu resistance than that of wild-type cells, supporting their functionality (Navarro et al., 2009). A summary illustration of these findings is shown in Figure 12.4.

When external Cu concentration increases, all of the Cu resistance determinants from *A. ferrooxidans* ATCC 23270 are expressed in higher levels to eliminate Cu from the periplasm or cytoplasm of the cells. This would require high levels of ATP to activate the metal efflux ATPases and the ATPases involved in the removal of protons generated by the Cus system to avoid cytoplasm acidification.

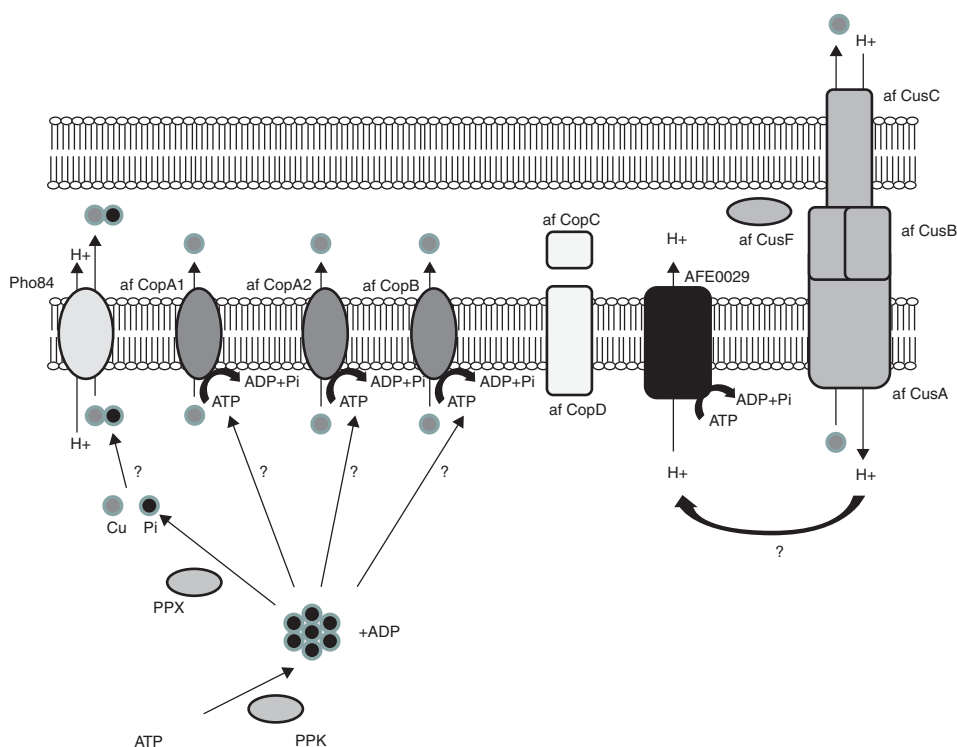


Figure 12.4. Possible working model for copper resistance in *A. ferrooxidans*.

A. ferrooxidans ATCC 53993 contains all the copper-resistance genes present in *A. ferrooxidans* ATCC 23270 that have been experimentally confirmed as being expressed in the presence of copper (Luo et al., 2008; Navarro et al., 2009; Orell et al., 2010). These ORFs have 100% identity between their corresponding DNA sequences. However, *A. ferrooxidans* ATCC 53993 contains several additional putative metal-resistance ORFs. These putative genes are clustered in a DNA region or genomic island (GI) that is absent in the genome of *A. ferrooxidans* strain ATCC 23270 (Cardenas et al., 2010; Orell et al., 2010). On the other hand, strain ATCC 23270 has a 300-kb region that is not complementary to the genome of strain ATCC 53993.

Bioinformatic analysis of the GI found exclusively in *A. ferrooxidans* ATCC 53993 predicted the presence of genes encoding heavy metal resistance determinants, such as mercury detoxification (*merA*), extrusion (*merC*), and the transcriptional regulator (*merR*), as well as a copper-translocating P-type ATPase. Among the other ORFs, a five-gene cluster potentially encoding arsenic-resistance components and heavy metal P-type ATPase was present (Cardenas et al., 2010). Further analysis indicated that the GI exclusive to strain ATCC 53993 was inserted after a possible gene coding for an enzyme related to tRNA metabolism and that this GI or indel contains an additional putative copper ATPase and a Cus system (Orellana and Jerez, 2011).

It is well known that, in general, GIs influence traits such as antibiotic resistance, symbiosis, fitness, and adaptation in pathogenic and environmental microorganisms (Dobrindt

et al., 2004). Genomic strain variations or interpopulation genetic exchanges have also been found in the acidophilic *Leptospirillum* group II by deep metagenomic genome sequence analysis (Simmons et al., 2008). Therefore, it is possible that horizontal gene transfers between these and other microorganisms are key elements to supplement metal resistance and possibly other properties, thus conferring adaptational advantages to these acidophiles. This idea was recently tested experimentally and it was found that *A. ferrooxidans* ATCC 53993 had a much higher resistance to CuSO_4 (>100 mM) than that of strain ATCC 23270 (<25 mM) (Orellana and Jerez, 2011). This behavior is probably explained by the presence of the additional copper-resistance genes in the GI of strain ATCC 53993.

It was expected that the additional multiplicity of possible copper-resistance determinants in the GI of *A. ferrooxidans* ATCC 53993 would confer on it a higher tolerance to the metal than that of strain ATCC 23270. Figure 12.5A illustrates the effect of 100 mM CuSO_4 on the growth of both *A. ferrooxidans* strains. Although the two strains have similar growth in the absence of copper (not shown), cell numbers of ATCC 23270 were reduced sevenfold when grown in 100 mM CuSO_4 , whereas those of ATCC 53993 were diminished only twofold. This additional capacity of strain ATCC 53993 to tolerate copper should confer on it an adaptational advantage when growing in a microbial consortium such as the one usually found in their habitat.

If a hypothetical reactor for bioleaching copper-containing mineral concentrates was inoculated initially with a mixture having equal proportions of each *A. ferrooxidans* strain, one would expect that ATCC 53993 would overgrow ATCC 23270 as copper is being extracted to the solution (Fig. 12.5B). A preliminary test for this idea was carried out by growing a mixture of approximately equivalent numbers of each strain in the absence or presence of copper. The relative proportions of each type of bacterium were estimated by means of qPCR by using strain-specific primers (Fig. 12.5C). These results clearly indicate that *A. ferrooxidans* ATCC 53993 is able to outgrow strain ATCC 23270 in the presence of 50 mM CuSO_4 (Orellana and Jerez, 2011).

Furthermore, an upregulation of the transcriptional expression of most of the additional Cu-resistance genes present in the GI of *A. ferrooxidans* ATCC 53993 was observed when cells were grown in the presence of increasing CuSO_4 concentrations. In addition, these genes were functional when expressed in *E. coli*, strongly supporting the functionality of the copper-resistance determinants present in the GI of *A. ferrooxidans* ATCC 53993 (Orellana and Jerez, 2011). These findings constitute the first experimental evidence for high copper resistance due to the expression of genes present in the GI of an acidophilic chemolithoautotrophic bacterium.

Thus, the reason for resistance to copper of two strains of the same acidophilic microorganism could be determined by slight differences in their genomes, generated by genetic exchange. These interpopulation exchanges may not only lead to changes in the capacities of the bacteria to adapt to their environment, but may also help to select the more fit microorganisms for industrial biomining operations.

12.4.2. Acidophilic archaeons

Related to archaeal Cu-resistance mechanisms, metal efflux pumps have been identified in the sequenced genomes of some members of the Archaea domain (Auernik et al., 2008; Bini, 2010; Orell et al., 2010). A Cu-resistance (*cop*) locus has been described in Archaea, which includes genes encoding a new type of archaeal transcriptional regulator

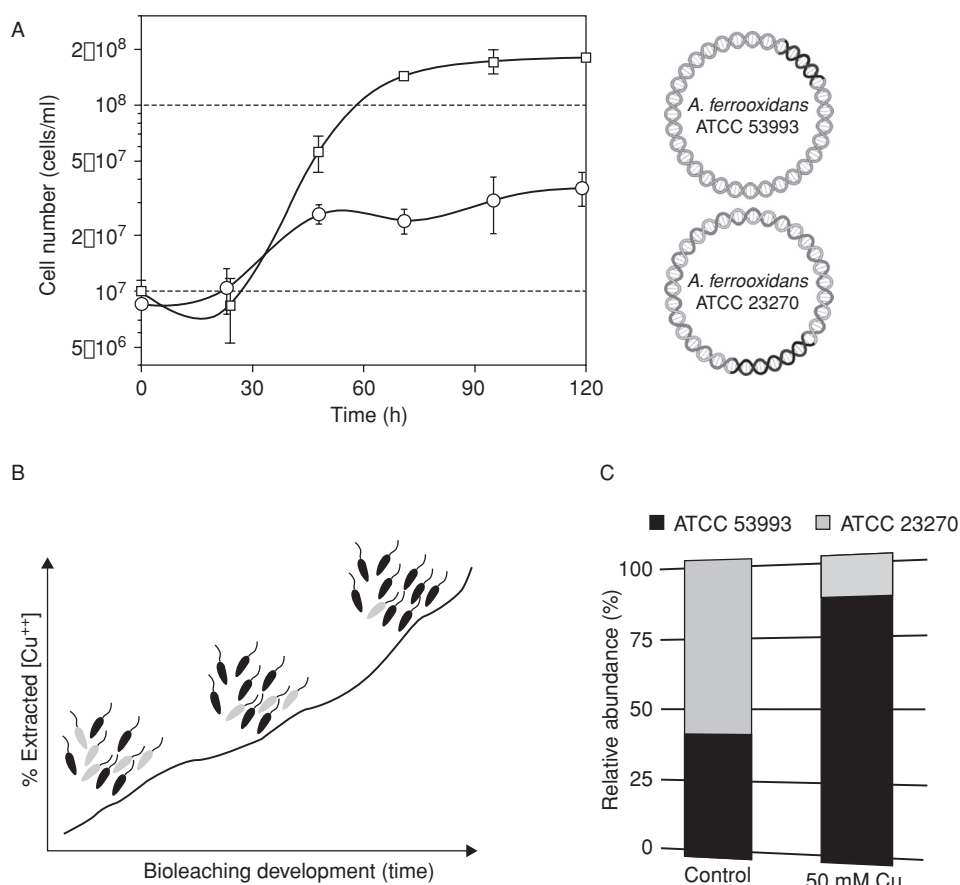


Figure 12.5. Effect of the presence of a genomic island in the capacity of *A. ferrooxidans* strains to tolerate high copper concentrations. (A) Both *A. ferrooxidans* ATCC 53993 and ATCC 23270 strains contain different exclusive genomic islands as seen in their respective genomic DNA representations. However, when each bacterium is grown in the presence of 100 mM copper sulfate, only ATCC 53993 grows in high cell numbers (upper curve). (B) Hypothetical bioreactor to bioleach a copper-containing mineral with an initial 1:1 mixture of strains ATCC 23270 (gray cells) and ATCC 53993 (black cells). (C) Quantitative RT-PCR determinations of the relative proportions of each *A. ferrooxidans* strain after their growth together in the absence (control) or presence of 50 mM copper.

(CopT), a putative metal-binding chaperone (CopM), and a putative Cu-transporting P-type ATPase (CopA) (Fig. 12.6). Recently, the same Cu-resistance mechanism was described in *Sulfolobus solfataricus* P2 and *Ferroplasma acidarmanus*. These transport systems would be operating for Cu efflux. The presence of duplicated putative genes encoding for Cu-ATPases (*copA1* and *copA2*) and two putative genes for metallochaperones (*copM1* and *copM2*) in the genomic DNA from *S. metallicus* has recently been demonstrated. The two Cu-ATPases are expressed when the archaeon is grown either in the presence of Cu or when using chalcopyrite (CuFeS₂) as an oxidizable substrate (Orell et al., 2010).

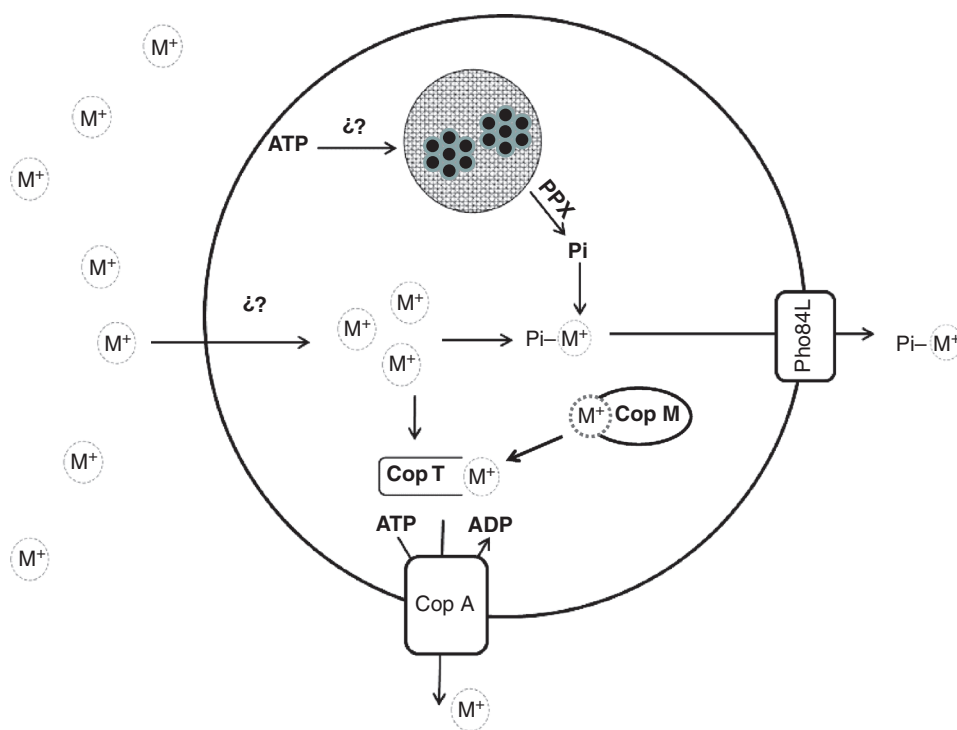


Figure 12.6. Possible working model for copper resistance in acidophilic archaeons.

The existence in acidophiles of genes similar to most of the Cu-resistance determinants contained in neutrophilic microorganisms does not completely explain the much higher metal resistance of the former microorganisms. One of the possible additional mechanisms proposed for metal resistance is the sequestration of metal cations with long polymers of inorganic polyphosphate (polyP) (Rao et al., 2009). PolyP is a linear polymer of hundreds of orthophosphate residues linked by phosphoanhydride bonds. The main enzyme involved in the biosynthesis of polyP is the polyphosphate kinase (PPK), which catalyzes the reversible conversion of the terminal phosphate of ATP into polyP. On the other hand, an exopolyphosphatase (PPX) is known to hydrolyze polyP liberating inorganic phosphate (Pi) (Keasling, 1997; Rao et al., 2009). PolyP is synthesized by PPK in *A. ferrooxidans* or other bacteria (or by a yet unknown equivalent archaeal enzyme) by using ATP (Figs. 12.4 and 12.6). Several biomining microorganisms have been shown to accumulate electron dense granules composed of polyP as seen in the bacteria *A. ferrooxidans*, *A. caldus*, and *A. thiooxidans* and in the archaeons *S. metallicus* and *Metallosphaera sedula*. A polyP-dependent system for Cu resistance has been proposed for *A. ferrooxidans* (Alvarez and Jerez, 2004) and *S. metallicus* (Remonsellez et al., 2006).

In excess of ADP generated by the use of cellular ATP, the reverse reaction of PPK synthesizes more ATP from polyP. In this way, the reserve polyP would also be supplying energy to the metal-detoxifying systems. A similar mechanism may also exist in *Sulfolobales* (Fig. 12.6) (Orell et al., 2010). Although this mechanism proposed for metal resistance

needs to be proven, it may eventually be functional in all polyP-accumulating biomining microorganisms (Orell et al., 2010) (Figs. 12.4 and 12.6).

A connection between pH homeostasis and metals homeostasis is not yet clear. However, it is a known fact that polyP has a high buffering capacity, being able to neutralize protons and metals that could be incorporated into the cytoplasm (Rao et al., 2009). The role we are proposing for polyP in eliminating Cu in acidophilic bacteria may be connected with its capacity to neutralize part of the protons generated by the Cus-like systems when Cu is being extruded to the outside of the cells in the acidophilic microorganisms (Fig. 12.4).

In summary, current knowledge indicates that key elements involved in the Cu resistance of *A. ferrooxidans* and similar microorganisms appear to be a wide repertoire of known Cu-resistance determinants and their duplication, the presence of novel Cu chaperones and an abundant reserve of inorganic polyP used in a polyP-based Cu-resistance system, a defensive response to oxidative stress, and the existence of genomic islands (Alvarez and Jerez, 2004; Orell et al., 2010; Orellana and Jerez, 2011).

It will be of great interest in future studies to establish whether all the remaining putative and hypothetical genes present in the GI of *A. ferrooxidans* ATCC 53993 have a role in conferring extra resistance not only to copper but also to several other metals found in bioleaching habitats. Furthermore, the characterization of metal-resistance determinants will not only contribute to our understanding of the mechanisms that acidophilic bioleaching microorganisms use to adapt to their extreme environment but eventually to improve biomining processes as well.

World copper production has increased steadily in the last 20 years, reaching close to 20 metric tons per year. About 20% of that copper is actually produced by hydrometallurgy (Watling et al., 2010; Jerez, 2011). Bioleaching is an important part of this production. Since the world demand for copper is growing, biohydrometallurgy for mine and production will continue to play an important role in mining industries (Brierley, 2009). New challenges and opportunities for improved processes will arise. Among these, the search for new extremophilic microorganisms with better capabilities to dissolve minerals at higher temperatures and with higher metal tolerances will be an important area of research.

Acknowledgments

Our work was supported by grant FONDECYT 1110214 and in part by ICM project P-05-001-F. I also thank C. Navarro and M. Rivero for their kind help with some of the drawings.

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BACTERIAL POLYMERS PRODUCED BY EXTREMOPHILES: BIOSYNTHESIS, CHARACTERIZATION, AND APPLICATIONS OF EXOPOLYSACCHARIDES

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13.1. INTRODUCTION

Extracellular polymeric substances (EPSs), produced by both prokaryotes (eubacteria and archaeobacteria) and eukaryotes (phytoplankton, fungi, and algae), are of topical research interest. The bacterial exopolysaccharides represent a wide range of chemical structures but have not yet acquired appreciable significance (Kumar, 2007). Metabolic engineering has recently been embraced as an effective tool for developing whole-cell biocatalysts in support of oligosaccharide and polysaccharide synthesis. Most impressive are the high product concentrations (up to 188 g/L) achieved through microbe-catalyzed synthesis (Ruffing and Chen, 2006). Exopolysaccharides (EPSs) make up a substantial component of the extracellular polymers surrounding most microbial cells in such extreme environments as antarctic ecosystems, saline lakes, geothermal springs, or deep-sea hydrothermal vents. Extremophiles have developed various adaptations, enabling them to compensate for the deleterious effects of such extreme conditions as high temperatures, salt, low pH, or high radiation levels. Among these adaptive strategies, EPS biosynthesis is one of the most common protective mechanisms. Chemically, EPSs are rich in high-molecular-mass polysaccharides (ranging from 1×10^5 to 3×10^5 Da) and have heteropolymeric composition. They have newfangled applications due to their unique properties. Accordingly,

EPSs have found multifarious applications in food, pharmaceutical, and other industrial fields. Hence, in this chapter we focus on bacterial extremophilic EPSs. Both extremophilic microorganisms and their EPSs suggest several biotechnological advantages, such as short fermentation processes for thermophiles and easily formed and stable emulsions of EPSs from halophiles. The precise role of the EPSs in EPS-producing bacteria, evident in different ecological niches, is dependent on the natural environment of the microorganism. Most of the functions ascribed to EPSs are protective in nature. The ability of a microorganism to surround itself with a highly hydrated EPS layer may provide it with protection against desiccation and predation by protozoans (Kumar et al., 2007). The increasing demand of natural polymers for various industrial applications has led to a vibrant interest in EPS production by microorganisms. In recent years, there has been a substantial interest in the isolation and identification of new microbial polysaccharides that might have innovative uses as emulsifiers, stabilizers, and gelling or texture-enhancing agents (Sutherland, 2001a).

13.2. EPS PRODUCED BY EXTREMOPHILIC BACTERIA

Many microorganisms (various species belonging to gram-positive and gram-negative bacteria, archaea, fungi, and algae) are known to produce extracellular polysaccharides or EPSs. This term was used for the first time by Sutherland in 1972 to describe carbohydrate polymers produced by marine bacteria with high molecular mass. As far as the microbial biodiversity is concerned, bacterial EPSs exhibit a wide range of chemical structures; several EPSs display high molecular mass as well as heteropolymeric composition. Bacterial EPSs usually occur in two forms: capsular polysaccharides, in which the polymers are bound covalently to the cell surface, and slime polysaccharides, which either remain attached (loosely bound) to the cell surface or are found in the extracellular medium as an amorphous matrix (Sutherland, 1982; Decho, 1990).

Most of the functions ascribed to EPSs are protective, and their specific roles are dependent on the ecological niches in which the producer microorganisms are bound to live. They could assist the microbial communities to endure extremes of temperature, salinity, and nutrient availability, creating a boundary between the bacterial cell and its immediate surroundings. Several EPSs produced by microorganisms from extreme habitats present biotechnological potentiality. By examining their structure and physicochemical characteristics, it is possible to gain insight into their commercial application, and some of them have already been employed in several industries, ranging from pharmaceutical to food-processing fields, as well as detoxification agents for the bioremediation of polluted areas from petrochemical oils (Poli et al., 2010). Actually, the successful application of EPSs depends largely on their physicochemical properties rather than on the sole yield achieved by the fermentation process.

Hypersaline environments, deep-sea hydrothermal vents, and volcanic and hydrothermal areas offer a new source of EPS-producing microorganisms as reported in Table 13.1. In addition, some archaea have also been investigated extensively as producers of polyhydroxyalkanoates, which could provide an internal reserve of carbon and energy (Poli et al., 2011). Several thermophiles, including *Methanococcus*, *Sulfolobus*, *Bacillus* spp., and more recently, *Thermotoga maritima* and *Thermococcus litoralis*, have been studied as producers of extracellular polysaccharides. *T. litoralis* is a heterotrophic facultative sulfur-dependent hyperthermophilic archaeon, isolated from a shallow submarine thermal spring with an

TABLE 13.1. Examples of Exopolysaccharides (EPSs) Produced by Extremophiles

Microorganism	Source Environment	EPS Name	Physical-Chemical Features	Chemical Composition	Suggested Ecologic Role	Proposed Biotechnological Application	References
<i>Archaea</i>							
<i>Sulfolobus solfataricus</i> strain MT4	Thermal ponds in Flegrean area, Agnano, Naples, Italy	EPS-MT4	Pentasaccharide repeating unit with high sulfate content	Glc/Man/GlcN/Gal (1.0 : 0.8 : 0.1 : 0.05)	Biofilm formation in response to thermoacidophile environmental conditions	Antiviral agent	Nicolaus et al., 1993
<i>Haloferax mediterranei</i> (ATCC 33500)	Salt ponds in Santa Pola, Alicante, Spain	EPS	MW > 100,000 Da; pseudoplastic behavior and viscosity resistant to extremes values of pH, temperature, and salinity	Man/Glc/Gal/amino sugars/uronic acids (Man as the major component); repeating unit: $\rightarrow 4$ - β -D-Glc pNAC- $(1 \rightarrow 6)$ - α -D-Manp- $(1 \rightarrow 4)$ - β -D-Glc pNAC-3-O-SO ₃ ⁻ - $(1 \rightarrow$ Man/Glc/Gal/Rha (0.6 : 0.3 : 1.0 : 0.3); repeating unit: α -D-Glc p $\rightarrow 4$)- α -D-Galp $\rightarrow 2$)- α -L-Rhap α -D-Galp $\rightarrow 4$)- β -D-Manp $\rightarrow 3,4$)- β -D-Manp $\rightarrow 2,3$)- β -D-Galp	Mucoidal layer enveloping cell colonies	Thickening agent and enhanced oil recovery	Parolis et al., 1996
<i>Haloferax gibbonsii</i> (ATCC 33959)	Solar saltern, Alicante, Spain	EPS	Heptasaccharide repeating unit with two sugar branches	Man/Glc/Gal/Rha (0.6 : 0.3 : 1.0 : 0.3); repeating unit: α -D-Glc p $\rightarrow 4$)- α -D-Galp $\rightarrow 2$)- α -L-Rhap α -D-Galp $\rightarrow 4$)- β -D-Manp $\rightarrow 3,4$)- β -D-Manp $\rightarrow 2,3$)- β -D-Galp	Mucoidal layer enveloping cell colonies	Thickening agent	Paramonov et al., 1998
<i>Halobacula japonica</i> strain T5	Marine saltern area in Monastir, Tunisia	EPS-T5	Pentasaccharide repeating unit with high sulfate content	GlcA/Man/Gal (1.0 : 0.6 : 0.3)	Assembling biofilm for nutrients accumulation and solid surface adhesion	Antiviral agent	Nicolaus et al., 1999

(continued)

TABLE 13.1. (Continued)

Microorganism	Source Environment	EPS Name	Physical-Chemical Features	Chemical Composition	Suggested Ecologic Role	Proposed Biotechnological Application	References
<i>Thermus aquaticus</i> strain YT-1	Hot spring in Yellowstone National Park, Wyoming	EPS-TA-1	MW = 500,000 Da; regular and stable structure due to the presence of <i>N</i> -acetylglactosamines, as the acetyl groups form helical aggregates at higher temperatures	<i>Thermophiles</i> Gal/ <i>N</i> -acetyl-GalN (1 : 1) repeating unit: galactofuranose→3,4- <i>N</i> -acetylglactosamine→3-galactopyranose→3-substituted <i>N</i> -acetylglactosamine	Protecting from environmental stress, such as high temperature	Vaccine adjuvant since EPS-TA-1 displayed to induce secretion of IL-6, TNF-α, and NO on murine macrophage and human monocyte-cell lines	Lin et al., 2011
<i>Thermotoga maritima</i>	Various geothermal heated locales on the seafloor	EPS	Flocculation of cellular aggregates on high-density cell cultures	Glu/Rib/Man (1.0 : 0.05 : 0.02)	Biofilm formation engulfing cell aggregates and enabling quorum sensing pathways in high-cell-density cocultures	Bioflocculating agent	Rinker et al., 2000; Johnson et al., 2005
<i>Geobacillus thermantarcticus</i>	Crater of Mount Melbourne, Antarctica	EPS-1	MW = 300,000 Da; high sulfate content	Man/Glc (1.0 : 0.7); heptasaccharide repeating unit constituted by four different α-D-Man and three different β-D-Glc	Protecting cells from environmental stress, such as high temperature	Bioemulsifier agent	Manca et al., 1996
<i>Geobacillus</i> sp. strain 4004	Sea sand in Maronti, Sant' Angelo, Ischia Island, Italy	EPS-3	MW = 1,000,000 Da; polydisperse nature and presence of elevated acidic residues; high gelling and divalent ion chelating capabilities	Gal/Man/GlcN/Ara (1.0 : 0.8 : 0.4 : 0.2); a pentasaccharide repeating unit constituted by two sugars with a gluco-galacto configuration and three with a manno configuration	Boundary between the bacterial cell and its immediate environment	Viscosifying and gelling agent	Nicolaus et al., 2002

<i>Geobacillus thermodenitrificans</i> strain B3-72	Vents of Vulcano Island, Italy	EPS-2	MW = 400,000 Da; a trisaccharide repeating unit constituted by sugars with a manno-pyranosidic configuration	Man/Glc (0.3 : 1.0)	Biofilm formation as a stress response to extreme environmental conditions	Adjuvant agent in equilibrating the immune response in viral diseases	Arena et al., 2009
<i>Geobacillus tepidamans</i> strain V264	Hot spring, Velingrad, Bulgaria	EPS-V264	MW > 1,000,000 Da; a galacto-glucan repeating unit with α -glycoside linkage	Glc/Gal/Fuc/Fru (1.0 : 0.07 : 0.04 : 0.02)	Mucoid layer enveloping cell colonies	Viscosity enhancer agent	Kambourova et al., 2009
<i>Halomonas maura</i> strain S-30	Saline soil from a solar saltern at Asilah, Morocco	Mauran	MW = 4.7×10^6 Da; high sulfate content; high metal-binding capacity; pseudoplastic behavior and viscosity resistant to high salt concentration and extreme pH values	<i>Halophiles</i> Man/Gal/Glc/GlcA (34.8 : 14.0 : 29.3 : 21.9)	Adhesion to surfaces, improvement of nutrient, and protection against environmental stress	Bioadsorbent in polluted environments and wastewater treatment	Arias et al., 2003
<i>Halomonas eurihalina</i> strain F2-7	Soil collected from a solar saltern at Alicante, Spain	EPS-V2-7	High sulfate content; viscosity increasing in acidic solutions; ability to emulsify hydrocarbons	Glc, Man, and Rha as principal neutral sugars	Pseudoplastic and rheological properties	Biosurfactant and bioemulsifier	Martínez-Checa et al., 2007
<i>Halomonas ventosae</i> strain A112	Saline soils in Jaén, southeastern Spain	EPS-A112	MW = 530,000 Da; high sulfate content; formation of stable emulsions and high metal-binding efficiency	Glc/Man/Gal (1.75 : 4.0 : 1.0)	Biofilm formation and metal chelation (biosorption)	Bioemulsifiers and bioremediation agent	Mata et al., 2006

(continued)

TABLE 13.1. (Continued)

Microorganism	Source Environment	EPS Name	Physical–Chemical Features	Chemical Composition	Suggested Ecologic Role	Proposed Biotechnological Application	References
<i>Halomonas antitartaricensis</i> strain FP35	Saline soils at Fuente de Piedra, an endorheic wetland in southern Spain	EPS-FP35	MW = 200,000 Da; high sulfate and phosphate content; high metal-binding efficiency	<i>Halophiles (continued)</i> Glc/Man/GalA (1.0 : 3.0 : 2.5)		Biofilm formation and metal chelation (biosorption)	Mata et al., 2006
<i>Halomonas</i> sp. strain AAD6	Çamaltı Saltern area in Turkey	Levan	MW > 1,000,000 Da; high specific viscosity; flocculating properties and high film-forming capability	Levan-type fructan; β -(2,6)-D-fructofuranosyl repeating unit	Biofilm formation and flocculating property under high-saline-environment conditions	Alternative cheap source of levan polymer, bioflocculant agent in treatment of industrial wastewaters, nanocarrier system for peptide and protein drug delivery	Poli et al., 2009; Kuçukasik et al., 2011; Sam et al., 2011; Sezer et al., 2011
<i>Halomonas alkaliarctica</i> strain CRSS	Salt lake at Cape Russell, Antarctica	EPS-B	Fructo-glucan configuration constituted by six different residues; high viscosity at low pH values and high NaCl concentration	Glc/Fru/GlcN/GalN (1.0:0.7:0.3:tr)	Mucoidal layer enveloping cell colonies	Viscosity control agent	Poli et al., 2007

^aMW, molecular weight; Glc, glucose; Fru, fructose; Gal, galactose; Man, mannose; Rha, rhamnose; Rib, ribose; Ara, arabinose; Fuc, fucose; GlcA, glucuronic acid; GalA, galacturonic acid; GlcN, glucosamine; GalN, galactosamine.

optimal growth temperature of 88°C. The sulfated EPS produced by *T. litoralis*, apparently involved in the formation of a biofilm enveloping cell colonies, contains mannose as the only monosaccharidic constituent, and this is a very peculiar feature for a prokaryote since the production of mannan-like constituents is typically reported for eukaryotes such as plants or yeasts (Rinker and Kelly, 1996).

Furthermore, in a study investigating the effect of carbon and nitrogen sources on growth dynamics and EPS production of *T. litoralis* and *T. maritima*, Rinker and Kelly (2000) found that *T. litoralis* was completely unable to utilize NH_4Cl as a nitrogen source, and its growth was even inhibited at certain levels of ammonium chloride. However, EPS production for both organisms was significant and increased at a rising dilution rate. In particular, *T. litoralis* yielded a twofold EPS production with respect to *T. maritima* under optimal growth conditions (0.32 and 0.1 g EPS/L at a dilution rate 0.4 h^{-1} for *T. litoralis* and *T. maritima*, respectively). In addition, in the presence of 1 g/L NH_4Cl , the EPS/CDW (cell dry weight) ratio (g/g) by *T. litoralis* was found to increase significantly at a rising dilution rate: 10 EPS/CDW at a dilution rate of 0.7 h^{-1} in the presence of 1 g/L NH_4Cl vs. 2 EPS/CDW at same dilution rate in the absence of NH_4Cl .

An additional example of EPS as a constituent of biofilm from archaea is referred to as *Archaeoglobus fulgidus*, the best characterized *Archaeoglobus* species. *A. fulgidus* is an anaerobic marine hyperthermophile that obtains energy with a dissimilator sulfate reduction by using H_2 , lactate, or pyruvate as the electron donor. The ability of *A. fulgidus* to colonize widely separated areas suggests that it has evolved mechanisms for surviving fluctuations in temperature, concentrations of nutrients, and potentially toxic compounds (Lapaglia and Hartzell, 1997). This archaeobacterium was found to form a biofilm in response to environmental stress conditions. The biofilm is a heterogeneous, morphologically variable structure containing proteins, polysaccharides, and metals. The biofilm production can be induced by nonphysiological extremes of pH and temperature, by high concentrations of metals, and by the addition of antibiotics, xenobiotics, or oxygen. Lapaglia and Hartzell (1997) demonstrated that cells within the biofilm showed an increased tolerance toward the toxic conditions of the external environment. Moreover, metals sequestered within the biofilm stimulated the growth of *A. fulgidus* cells in a metal-depleted medium, suggesting that cells may produce biofilm as a mechanism for concentrating cells, which resulting in their attachment to external surfaces as a protective barrier and at the same time acting as a reserve of nutrients. Since similar biofilms are formed by *Archaeoglobus profundus*, *Methanococcus jannaschii*, and *Methanobacterium thermoautotrophicum*, the biofilm formation might be a common stress-response mechanism among the archaea (Hartzell et al., 1999).

The extreme thermoacidophile archaeon *Sulfolobus solfataricus* strains MT4 and MT3 were observed to produce a sulfated EPS containing glucose, mannose, glucosamine, and galactose under optimal growth conditions (Table 13.1). Maximum EPS production was reached during the stationary phase of growth, and the yields obtained were 8.4 and 7.0 mg/L for the MT4 and MT3 strains, respectively (Nicolaus et al., 1993).

In a screening program launched to find new polyhydroxyalkanoate and EPS producers, Nicolaus et al. (1999) isolated three obligate halophilic microorganisms (the T5, T6, T7 strains) from an unexplored site in Tunisia (Monastir). All the isolates had polar lipid patterns, which is a peculiar characteristic of species belonging to the genus *Haloarcula*; in particular, strain T5 was identified as a new strain of *H. japonica* by DNA–DNA hybridization analysis (Nicolaus et al., 1999). These strains were grown on a minimal medium

containing glucose as the sole carbon source and they were shown to produce sulfated extracellular polysaccharides under such conditions. Sugar analysis of the EPS produced by strain T5 revealed mannose, galactose, and glucuronic acid, in the relative proportion 0.6:0.3:1.0, respectively, as principal constituents (Table 13.1). Moreover, sugar analysis of crude EPSs of both strains T6 and T7 yielded as principal constituents mannose, galactose, and glucose in the same relative proportion of 1.0:0.2:0.2.

Haloferax mediterranei, an EPS-producing archaeobacterium, was initially described by Anton et al. (1988) as a producer of an exocellular polymeric substance that gave a typical mucous character to the colonies and was responsible for the appearance of a superficial layer in an unshaken liquid medium. The polymer, identified as a heteropolysaccharide containing mannose as the major component, also contained glucose, galactose, an unidentified neutral sugar, amino sugars, uronic acids, and a considerable amount of sulfate as well. The structure of the repeating unit of this polymer was subsequently determined by Parolis et al. (1996) by a combination of glycosylation, methylation, sulfate analysis, periodate oxidation, and ^1D - and ^2D -NMR spectroscopic analysis of the native and periodate-oxidized/reduced polysaccharides and was reported as $\rightarrow 4\text{-}\beta\text{-D-GlcpNAcA-(1}\rightarrow 6\text{)-}\alpha\text{-D-Manp-(1}\rightarrow 4\text{)-}\beta\text{-D-GlcpNAcA-3-O-SO}_3^--(1\rightarrow$ (Table 13.1). Paramanov et al. (1998) reported the structure of the neutral EPS isolated from *H. gibbonsii* ATCC 33959, determined by means of NOESY and HMBC-NMR experiments (Table 13.1). The polysaccharide contained mannose, glucose, galactose, and rhamnose in the ratio 0.6:0.3:1.0:0.3. The substitution patterns of the sugar residues were deduced from the methylation analysis, which indicated a heptasaccharide repeating unit containing two branches.

Parolis et al. (1999) reported the structure of a linear, acidic EPS isolated from *H. denitrificans*, an extremely halophilic organism that grows in the presence of salt concentrations ranging from 1.5 to 4.5 M. This archaeon is aerobic, highly pleomorphic, and produces orange-red colonies. The sugar residues in the repeating unit of the polysaccharide were identified as $\rightarrow 4\text{-}\beta\text{-D-GlcpA2,3NAc-(1}\rightarrow 4\text{)-}\beta\text{-D-GlcpA2,3NAc-(1}\rightarrow 4\text{)-}\alpha\text{-D-GlcpA2,3NAc-(1}\rightarrow 3\text{)-}\alpha\text{-D-Galp-(1}\rightarrow$, where D-GlcpA2,3NAc is 2,3-diacetamido-2,3-dideoxy-D-glucopyranosiduronic acid.

In the domain Bacteria, several thermophilic microorganisms are good producers of large amounts of EPSs. Thermophilic bacteria belonging to the genus *Geobacillus* have been isolated from shallow marine hydrothermal vents of flegrean area in Italy and characterized as EPS producers, as reported in Table 13.1 (Nicolaus et al., 2002). Actually, an EPS-producing thermophilic strain, strain 4004, belonging to the genus *Geobacillus*, was isolated from sediment samples in a marine hot spring near the seashore of Maronti (Ischia Island, Italy). The EPS produced by strain 4004 in the sucrose medium was isolated via ethanol precipitation of the cell-free medium. The ^1H - and ^{13}C -NMR spectra of the purified polymer displayed five different residues within the repeating saccharidic unit: two of them with a gluco-galacto configuration and three with a mannan configuration. One of the residues is an acetamido-sugar, and at least one uronic acid is present. Sugar analysis was performed on a hydrolyzed polysaccharide fraction, and the EPS was reported to be composed of galactose, mannose, glucosamine, and arabinose in the relative ratio 1.0:0.8:0.4:0.2, respectively (Table 13.1).

Furthermore, marine shallow hydrothermal vents around the volcanic Eolian Islands close to Sicilian coasts (Italy) represent accessible fields for the isolation of thermophilic bacteria. Previous studies described diversity and distribution of bacterial communities within deep and shallow hydrothermal systems at Porto di Levante, Vulcano, revealing the

presence of chemosynthetic, thermophilic, archaeal, and bacterial strains (Gugliandolo and Maugeri, 1998). In addition, a thermophilic aerobic microorganism able to produce two exocellular polysaccharides (EPS-1 and EPS-2) was isolated from seawater of a shallow hydrothermal vent at Vulcano Island (Eolian Islands, Italy). This new eolian thermophilic isolate was identified as *Bacillus thermodenitrificans* strain B3-72. In particular, hydrolysis of EPS-2 yielded mannose and glucose as principal constituents in the relative ratio 1.0 : 0.2. The ^{13}C - and ^1H -NMR spectra of this polymer revealed a trisaccharide repeating unit constituted essentially of sugars having a mannopyranosidic configuration (Table 13.1).

In subsequent experiments, the immunomodulatory and antiviral effects of the EPS-2 produced by *B. thermodenitrificans* were evaluated, since polysaccharides with high molecular mass exhibited immunogenic activity (Guezennec et al., 1994). Indeed, sulfated EPSs are known to interfere with the absorption and penetration of viruses into host cells and to inhibit various retroviral reverse transcriptases (Hayashi et al., 1996). EPS-2 from *B. thermodenitrificans* was also able to obstruct HSV-2 (herpes simplex virus type 2) replication in human peripheral blood mononuclear cells (PBMCs). Actually, high levels of IFN- α , IL-12, IFN- γ , TNF- α , and IL-18 (IFN, interferons; TNF, tumor necrosis factor; IL, interleukine) were detected in supernatants of PBMCs treated with EPS-2; additionally, the stimulating effect of the inflammatory response was found to be dose dependent. Those results highlight the potential role of EPS-2 from *B. thermodenitrificans* as adjuvant in equilibrating the immune response during viral infection and that the immunological disorders determined by HSV-2 could be partially restored by treatment with EPS (Arena et al., 2009).

Another EPS with immunomodulatory properties was studied from the thermotolerant *Bacillus licheniformis* strain B3-15, isolated from water of a shallow marine hot spring at Vulcano Island. The EPS was found to possess a tetrasaccharide repeating unit constituted essentially of sugars having a manno-pyranosidic configuration (Maugeri et al., 2002). Solutions of the EPS from *B. licheniformis* exhibited a marked, dose-dependent decrease in HSV-2 replication on in vitro cultures of PBMCs (Arena et al., 2006). To assess whether the antiviral activity induced by the EPS in PBMC could be related to an immunomodulatory mechanism, the production of various cytokines (e.g., IFN- α , IL-12, IFN- γ , TNF- α , and IL-18) involved in the immune response toward virus infection was evaluated. As a result, high levels of all these cytokines were detected in supernatants from PBMCs treated with the EPS. On the other hand, IL-4, a strong hallmark of Th2 response, was not detected in any of the supernatants tested. According to the data collected, the effect of the EPS was dose dependent when PBMCs were treated with the EPS and simultaneously infected with HSV-2, and the cytokine production that resulted was down-regulated. Consequently, those results suggest that the EPS produced by *B. licheniformis* may contribute to improving immune surveillance of PBMCs toward virus infection, eliciting a therapeutic Th1-like response in clinical cases of viral diseases as well as in immune-compromized hosts.

Lin et al. (2011) isolated a novel extracellular polysaccharide (EPS TA-1) from the biofilm of *Thermus aquaticus* strain YT-1. They found that EPS TA-1 induces murine macrophage and human monocyte cell lines to secrete IL-6, TNF- α , and NO. The polysaccharide is composed of tetrasaccharide repeating units of galactofuranose, galactopyranose, and *N*-acetylgalactosamine (1.0 : 1.0 : 2.0) and lacked acidic sugars (Table 13.1).

Bacillus thermantarcticus, proposed at first with the name of the genus "Bacillus" by Nicolaus et al. (1996), and recently transferred to the genus *Geobacillus* as *G. thermantarcticus* (Coorevits et al., 2011), is able to produce two different sulfated polysaccharides (EPS-1

and EPS-2) that confer a typical mucous character to the colonies. Nuclear magnetic resonance (NMR) spectra confirmed that EPS-1 was a heteropolysaccharide, whose repeating unit comprises four different α -D-mannoses and three different β -D-glucoses. It seems to be close to some xantan polymers. EPS-2 displayed a mannan configuration with four different α -D-mannoses in the repeating unit (Table 13.1).

A glucan with high molecular mass ($>1,000,000$ Da) was isolated from a thermophilic strain, *G. tepidamans* strain V264, from a Velingrad, Bulgaria hot spring. Maltose was found to be the most appropriate carbon source for EPS production. Maximum EPS production was reached in the early stationary phase of growth. The purified EPS was very stable at high temperature, displaying a total degradation at 280°C . The chemical composition of the biopolymer, determined on the hydrolyzed EPS with a high-pressure anion exchange-pulsed amperometric detector (HPAE-PAD), showed glucose as its major component (Table 13.1).

Hypersaline environments, found in a wide variety of aquatic and terrestrial ecosystems, are inhabited by halotolerant microorganisms as well as halophilic microorganisms (0.5 to 2.5 M NaCl) and extreme halophiles (>2.5 M NaCl). Moderate and extreme halophiles have been isolated from hypersaline ecosystems (salt lakes, marine salterns, and saline soils) and also from alkaline ecosystems (alkaline lakes). Halophilic microorganisms have developed various biochemical strategies to survive under high-saline conditions, including compatible solute synthesis to preserve cell structure and function. Their products, such as ectoine, bacteriorhodopsins, EPSs, hydrolases, and biosurfactants, are noticeably of industrial interest (Poli et al., 2010). Indeed, the genus *Halomonas* has received increasing interest, as several *Halomonas* species are able to produce significant quantities of EPS with high surface activity and/or rheological properties (Martínez-Checa et al., 2002). Some species belonging to the genus *Halomonas* are able to produce EPSs: *H. rifensis* (Amjres et al., 2011), *H. maura* (Arias et al., 2003), *H. alkaliphila* (Romano et al., 2006), *H. alkaliantartica* (Poli et al., 2004, 2007), *H. ventosae* (Martínez-Cánovas et al., 2004a), *H. anticariensis* (Martínez-Cánovas et al., 2004b), *H. almeriensis* (Martínez-Checa et al., 2005), *H. cerina* (Gonzales-Domenech et al., 2008a), *H. nitroreducens* (Gonzalez-Domenech et al., 2008b), *H. caseinilytica* (Wu, Y.H. et al., 2008), *H. daqingensis* (Wu, G. et al., 2008), *H. eurihalina* (Martínez-Checa et al., 2002), *H. fontilapidosi* (Gonzalez-Domenech et al., 2009), and *H. sabkhae* (Kharroub et al., 2008). For most of them, only their EPS production capability as a phenotypic characteristic to classify novel species has been identified. For some of them, the EPS sugar composition is also known, as reported in Table 13.1. In particular, the EPS of *H. maura* was shown to contain mannose, galactose, and glucose in the relative proportion 1.0:0.6:0.2 (Arias et al., 2003); in the EPS from *H. anticariensis* strain FP36, glucose, mannose, and galacturonic acid were reported (Mata et al., 2006); the EPS of *H. ventosae* strain A116 revealed the presence of glucose, mannose, and galactose (Mata et al., 2006); and the EPS of *H. alkaliantartica* presented glucose, fructose, glucosamine, and galactosamine in the relative proportion 1.0:0.7:0.3:0.2, when the strain was grown in sodium acetate as the sole carbon source (Poli et al., 2004). Moreover, a novel halophile, from soil samples taken from *Halomonas* sp. strain AAD6, isolated the Çamaltı Saltern area in Turkey, was found to produce high levels of EPS in presence of sucrose in the media, and fermentation growths under batch and bioreactor conditions yielded 1.073 and 1.844 g/L, respectively (Poli et al., 2009). Sugar composition analysis, methylation, and NMR studies of the EPS indicated that the repeating unit was composed of β -(2,6)-D-fructofuranosyl residues. Hence, this work displayed for the first time a *Halomonas* sp. as a levan producer

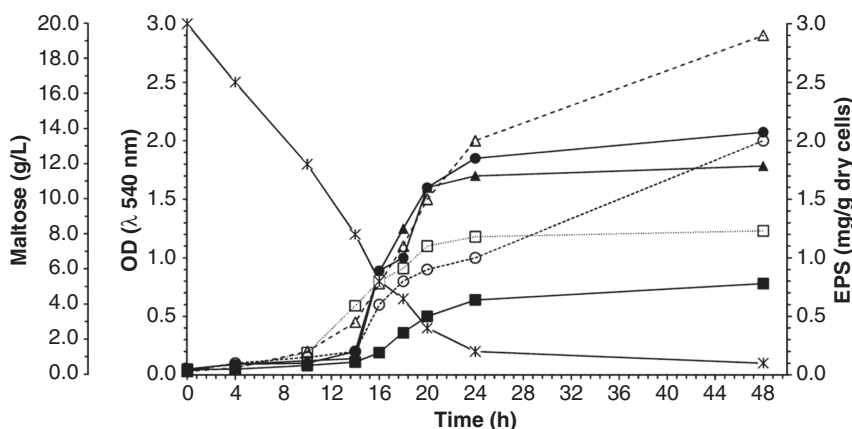


Figure 13.1. Growth-associated EPS biosynthesis by *Halomonas alkaliantarctica* strain CRSS as reported in Poli et al. (2004): biomass on minimal media C with acetate (■), EPS production on minimal media C with acetate (□), biomass on complex media A (●), EPS production on complex media A (○), biomass on complex media B with maltose (▲), EPS production on complex media B with maltose (Δ), maltose (*).

microorganism. Indeed, biocompatibility studies showed that this EPS did not affect cellular viability and proliferation of osteoblasts and murine macrophages, and the protective effect of the EPS against the toxic activity of avarol implied its additional use as an anticytotoxic agent. Therefore, *Halomonas* sp. strain AAD6 could represent an alternative cheap source of levan polymer when grown on definite media, hypothesizing its larger employment in industrial applications as a nonpathogenic microorganism (Kuçukasik et al., 2011), as a biofloculant agent in the treatment of industrial wastewaters (Sam et al., 2011), and in a nanocarrier system for peptide and protein drug delivery (Sezer et al., 2011) according to its high film-forming capability (Sima et al., 2011).

H. alkaliantarctica strain CRSS was isolated from salt sediments near the salt lake at Cape Russell, Antarctica (Poli et al., 2004, 2007). The strain CRSS grew aerobically in a complex medium containing 100 g/L NaCl. The highest EPS production was achieved when the complex medium was emended with maltose (20 g/L) as reported in Figure 13.1. Among various carbon sources tested on minimal medium, sodium acetate was established to be the most efficient in terms of biomass yield and EPS production. The hydrolyzed EPS presented a monosaccharide composition of glucose, fructose, glucosamine, and galactosamine in the relative proportion of 1.0 : 0.7 : 0.3 : tr, respectively. The ^1H - and ^{13}C -NMR spectra showed the presence of six different residues in the repetitive saccharidic unit, five of them with α -configuration and one with a mannan configuration indicating a complex primary structure of the biopolymer.

13.3. EXAMPLES OF PROPOSED EPS BIOSYNTHESIS FROM EXTREMOPHILES

Extensive progress has been made in recent years in determining the biosynthetic and genetic mechanisms involved in the synthesis of EPSs. The biosynthesis of most EPSs

closely resembles the process by which the bacterial cell wall polymers, peptidoglycan and lipopolysaccharide are formed. Indeed, these three types of macromolecules share the feature of being synthesized at the cell membrane and being exported to the final site, the external layer of the cytoplasmic membrane. The only exceptions are levans, alternans and dextrans, which are synthesized by an extracellular process (Vanhooren and Vandamme, 1998).

The enzymes involved in EPS synthesis are located in different regions of the microbial cell and may be classified into four groups.

- *Group I.* The first group of enzymes is found intracellularly and is involved in many other cell metabolic processes. One of them is hexokinase, which is involved in the phosphorylation of glucose (Glc) to glucose-6-phosphate (Glc-6-P), and the second enzyme, phosphoglucomutase, converts Glc-6-P to glucose-1-phosphate (Glc-1-P).
- *Group II.* The second group of enzymes is supposed to be intracellular. One of them includes uridine diphosphate–glucose pyrophosphorylase (UDP-glucose pyrophosphorylase). This enzyme catalyzes the conversion of Glc-1-P to uridine diphosphate glucose (UDP-Glc), which is the key molecule in EPS synthesis.
- *Group III.* This group of enzymes is located in the cell periplasmic membrane and referred to as glycosyltransferases. They transfer the sugar nucleotides UDP-Glc or UDP-Gal and/or UDP-GlcA to a repeating unit attached to glycosyl carrier lipid. The carrier lipid is identified as an isoprenoid alcohol, and its terminal alcohol group is attached to a monosaccharide residue through a pyrophosphate bridge.
- *Group IV.* This group of enzymes, situated outside the cell membrane and the cell wall, is presumably involved in the polymerization of the macromolecules. The EPS is then extruded from the cell surface to form a loose slime or a well-attached polysaccharide capsule surrounding the cell (Margaritis and Pace, 1985).

The synthesis of EPS is an intracellular process involving nucleoside diphosphate sugars. This process involves a cassette of genes, whose products are responsible for acylation and the addition of individual sugars to isoprenoid lipid acceptors. The repeating units are polymerized on the carrier lipids and excreted into the extracellular environment, as reported in Figure 13.2. Typically, a gene sequence on the order of 12 to 17 kb may be required depending on the complexity of the polysaccharide.

Considerable similarity among the gene products from different polysaccharide-synthesizing systems has been noted. Although less is known about the polymerization and excretion mechanisms, an ABC transporter is almost certainly involved in those systems that have been best characterized (Sutherland, 2001b). In the case of *Xanthomonas campestris*, enzymes needed for the formation of precursors, which are not specifically associated with EPS production, apparently seem to be under separate control, whereas gene products required exclusively for xanthan production occur in the linked group of genes.

The mechanisms involved in assembly, polymerization, and translocation across the outer membrane in gram-negative bacteria follow a pathway similar to that of some capsular EPSs produced by *Escherichia coli* groups 1 and 4 (Rahn et al., 1999) and *Klebsiella pneumoniae* (Arakawa et al., 1995), and of extracellular polysaccharides, including those produced by *Erwinia* spp. (Bugert and Geider, 1995), *Methylobacillus* sp. strain 12S (Yoshida et al., 2003), *Rhizobium* spp. (Reuber and Walker, 1993), and *X. campestris* (Katzen et al., 1998). Undecaprenol-pyrophosphate-linked oligosaccharide

repeating units are formed at the cytoplasmic face of the inner membrane, transported through this membrane by a Wzx-protein-dependent process, and then polymerized by a mechanism involving a Wzy protein. Some of the polysaccharide-biosynthesis gene clusters have been cloned and sequenced and found to form long operons with similarities in their genetic organization. In general, the first three genes, which are necessary for high-level polymerization and surface assembly, are conserved in the above-mentioned microorganisms: *wza* (encoding an outer-membrane protein), *wzb* (encoding an acid phosphatase), and *wzc* (encoding an inner-membrane tyrosine autokinase) (Drummelsmith and Whitfield, 2000).

Analysis of the flanking regions of a mini-Tn5 insertion site in an EPS-deficient mutant of *Halomonas maura* strain TK71 led to the identification of five ORFs (*epsABCDJ*), which form part of a gene cluster (*eps*) with the same structural organization as others involved in the biosynthesis of group 1 capsules and some EPSs. The possibility that mauran might be synthesized via a Wzy-like biosynthesis system, just as it is for many other polysaccharides, is reported by Arco et al. (2005). This hypothesis is supported by the fact that EpsJ is homologous with some members of the PST-exporter-protein family, which seems to function together with each OMA-PCP pair in polysaccharide transport in gram-negative bacteria, transferring the assembled lipid-linked repeating units from the cytoplasmic membrane to the periplasmic space (Arco et al., 2005).

Methylobacillus sp. strain 12S produces the EPS, methanolan, composed of glucose, mannose, and galactose. Yoshida et al. (2003) demonstrated that 24 ORFs flanking a Tn5 insertion site in an EPS-deficient mutant and 21 genes (*epsCBAKLDEFGHIJMNOPQRSTU*) were predicted to participate in methanolan synthesis on the basis of the features of the primary sequence. Gene disruption analyses revealed that *epsABCEFGIJNOP* and *epsR* are required for methanolan synthesis, whereas *epsKD* and *epsH* are not essential. EpsFG and EpsE showed homology with Wzc (chain-length regulator) and Wza (export protein) of group 1 capsule-producing *E. coli*, suggesting that methanolan was synthesized via a Wzy-like biosynthesis system.

The genomes of three model extreme thermophiles—an archaeon, *Pyrococcus furiosus* (T_{opt} of 98°C), and two bacteria, *Thermotoga maritima* (T_{opt} of 80°C) and *Caldicellulosiruptor saccharolyticus* (T_{opt} of 70°C)—encode numerous carbohydrate-active enzymes, many of which have been biochemically characterized in their native or recombinant forms. In addition to their voracious appetite for polysaccharide degradation, VanFossen et al. (2008) also reported the polysaccharide production for extremely thermophilic fermentative anaerobes. *T. maritima* generates exopolysaccharides that support biofilm formation, a process that appears to be driven by intra- and interspecies interactions. Various thermoacidophilic archaea, including members of the genera *Thermococcus* and *Sulfolobus*, have been observed to accumulate intracellular polysaccharides, such as glycogen, when grown with sucrose as a carbon source (Konig et al., 1982). Moreover, *Sulfolobus* (Nicolaus et al., 1993), *Thermococcus* (Rinker et al., 1996), and *Thermotoga* (Rinker et al., 2000) species produce EPSs. As an additional cellular function, EPS mediates adhesion, allowing cells to attach to other cells and solid surfaces, forming a matrix of cells, with the EPS commonly referred to as a biofilm. Pure cultures of *T. maritima* (Rinker et al., 2000), *Archaeoglobus fulgidus* (Hartzell et al., 1999), and *Thermococcus litoralis* (Rinker et al., 1996) formed significant biofilms under a variety of experimental conditions (VanFossen et al., 2008). The basics of thermophilic biofilm formation appear to proceed in much the same manner as for mesophiles and involve the initial attachment of cells to a solid support, production

of EPS, early biofilm development, mature biofilm development, and the subsequent detachment of cells (Kolter and Losick, 1998). The key genes were found to be involved in chemotaxis, motility, EPS synthesis, and stress response. The expression of these specific genes has been linked to quorum-sensing behavior in a number of bacterial species.

Johnson et al. (2005) proposed the pathway for EPS production in *T. maritima* (Fig. 13.2). They demonstrated the population density-dependent regulation of EPS formation. The cocultivation of *T. maritima* and *Methanococcus jannaschii* resulted in a fivefold increase in *T. maritima* cell densities when compared to monocultures, as well as an increase in EPS formation. Transcriptional response data from the cocultivation experiments, in conjunction with previous studies of EPS production in mesophilic bacteria, provided the basis for the proposed EPS production pathway reported in Figure 13.2.

Several studies have shown that a number of peptides, despite lacking readily identifiable structural features, are involved in inter- and intraspecies signaling, antimicrobial behavior, surface adhesion, and substrate acquisition. Understanding the details of EPS formation in hyperthermophilic microorganisms will require determining which signals are expressed by key genes and how cells respond to such signals.

13.4. PHYSICOCHEMICAL INVESTIGATIONS FOR POTENTIAL APPLICATIONS

Studies of the EPS structure are crucial not only to understand its physicochemical and biological properties, but also for the exploitation of EPS-producing microorganisms in industrial or medical applications. Several chemical and physical techniques are used to determine the primary structure of EPSs (Nicolaus et al., 2010). Chemical degradation and derivatization, combined with chromatographic methods, often coupled with mass spectrometry, are used to determine the sugar composition, together with the absolute configuration, their positions of substitution, and the substituent composition. The main steps involved in the recovery, purification, and structural characterization of microbial EPSs are shown in Table 13.2.

Nuclear magnetic resonance, in particular two-dimensional ^1H - and ^{13}C -NMR, is the most powerful technique to use to obtain information about the nature and configuration of sugar residues, their interconnectivity, and the nature and location of substituents, thus ultimately determining the sequence of the repeating unit. Usually, chemical or enzymatic fractionation of the polysaccharide is required to produce smaller fragments that are more easily analyzed by NMR or mass spectrometry. In addition to these classical chemical procedures, new and more powerful tools such as Raman microspectroscopy and atomic force microscopy techniques have been applied to investigate the cultivation-time dependence of bacterial cellular surface biopolymers at the single cell level (Lucas et al., 2009).

The producers of microbial EPSs with industrial value are mainly mesophilic microorganisms. Information concerning polysaccharides produced by extremophilic prokaryotes is scarce, and exploitation of their unusual properties is a question for future industrial processes. It is now widely accepted that extremophilic microorganisms will provide a valuable resource not only for exploitation in novel biotechnological processes but also as models for investigating how biomolecules are stabilized when subjected to extreme conditions. Compared with other EPS producers in the biosphere, microbial isolates from extreme environments offer a great variety in the chemical and physical properties of their EPSs.

TABLE 13.2. Procedures in EPS Characterization

<i>Downstream Process</i>	
1. <i>Separation of fermentation medium from microbial biomass</i>	The process is usually accelerated by high-speed centrifugation force.
2. <i>Ultrafiltration of the supernatant</i>	Suspended solids and particles are retained onto the filter; thus, the EPS concentration increases.
3. <i>Precipitation with organic solvents</i>	The high-molecular-mass compounds, including exopolysaccharides, are usually precipitated with organic solvents at low temperature (-20°C) overnight. The EPS precipitated is pelleted by centrifugation and dissolved in hot water. Previous washing steps are typically required.
4. <i>Dialysis</i>	The solution containing EPS is dialyzed with semipermeable membranes against water.
5. <i>Lyophilization</i>	EPS solution is freeze-dried, yielding the raw EPS.
<i>Physical–Chemical Characterization Steps</i>	
1. <i>Gel filtration chromatography</i>	Previous size-exclusion chromatographic steps are typically required to get rid of impurities and yield a homogeneous sample. In particular, the fractions with high carbohydrate content are collected and then utilized for characterization analyses.
2. <i>Ion-exchange chromatography</i>	Anion-exchange chromatography is typically required, especially for the purification of anion-charged molecules such as acidic exopolysaccharides, and enriched carbohydrate fractions are submitted for further characterization analyses.
3. <i>Physical–chemical composition</i>	<ul style="list-style-type: none"> • <i>Molecular mass</i> Determination of the average molecular size of the polymers. • <i>Total carbohydrate, protein, and nucleic acid content</i> Usually detected spectrophotometrically and expressed as a percentage. • <i>Optical rotation</i> Capability of the polymer solution to rotate the plane-polarized light within a specific angle α.
4. <i>Fine structure definition</i>	<ul style="list-style-type: none"> • <i>FT-IR (Fourier-transformed infrared spectroscopy)</i> Detection of chemical substituents (e.g., sulfate, acetyl, pyruvate groups). • <i>Monosaccharide composition</i> Acidic hydrolysis coupled with high-pressure anion-exchange chromatography. • <i>Raman microspectroscopy combined with atomic force microscopy (AFM)</i> In situ nondestructive characterization of the structure and the physical properties of individual nanostructures. • <i>Chemical or enzymatic degradation and derivatization</i> Polysaccharide fragments are analyzed successively by: <ul style="list-style-type: none"> • <i>GLC-MS (gas–liquid chromatography coupled with mass spectrometry)</i> Determination of sugar composition, absolute configuration, presence and position of substituents. • <i>NMR (nuclear magnetic resonance)</i> Identification of anomeric linkage between sugar components by mono- and bidimensional ^1H and ^{13}C.

Several EPSs produced by microbes from these extreme environments embody promising biotechnological applications (Table 13.1). By examining the chemical characteristics of these carbohydrate polymers, it is possible to begin to understand the ecological role of these natural products as well as to acquire new insight into their commercial potential. In fact, their specific rheological properties, either in the presence or absence of monovalent and divalent ions, biological activities, metal-binding capabilities, and novel chemical compositions imply that these EPSs are expected to find many applications in the very next future (Guezennec, 2003; Mancuso Nichols et al., 2004; Poli et al., 2004).

Acknowledgment

The work was funded partially by the Great Relevance Project, Agreement Italy–Turkey (MAE–Ministry of Italian Foreign).

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BIOMEDICAL APPLICATIONS OF EXOPOLYSACCHARIDES PRODUCED BY MICROORGANISMS ISOLATED FROM EXTREME ENVIRONMENTS

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14.1. INTRODUCTION

Polysaccharides constitute part of the outer envelope of many microorganisms. These polymers, in addition to their structural function, play a protective role and participate in the process of adhesion to both biological and inert surfaces. Some of them form capsules that are intimately associated with the cell surface, but others are slimes, which are eventually excreted into the environment.

The term *exopolysaccharides* (EPSs), proposed by Sutherland in 1972, is used to describe polysaccharides found in the medium outside microbial cells, generally participating in the formation of microbial aggregates (Geesey, 1982). The majority of microorganisms live in natural environments in aggregated forms known as *biofilms*. Biofilms are widely distributed throughout soils and aquatic environments, on plant and animal tissues, as well as in artificial systems such as filters, reservoirs, plumbing, heaters, and medical prostheses. They contain mixed populations of microorganisms embedded in a matrix of extracellular polymeric substances composed mainly of water and extracellular polysaccharides, or

exopolysaccharides. They also include proteins, lipids, and nucleic acids derived from microbial lysis (Costerton et al., 1987; Wingender et al., 1999; Flemming and Wingender, 2001; Sutherland, 2001a,c).

EPSs are widely distributed, especially among both saprophytic and pathogenic species of prokaryotes, and are also found in many microalgae, although they are less common among fungi (Sutherland, 1990; Wingender et al., 1999).

14.2. CHEMICAL COMPOSITION AND STRUCTURE OF EPSs

Exopolysaccharides are complex molecules formed by the repetition of one or more types of sugar linked by glycosidic bonds, the most common of which are glucose, galactose, and mannose. Other neutral sugars, such as rhamnose and fucose, some uronic acids, and amino sugars are also frequently present (Sutherland, 1990; Freitas et al., 2011). Eukaryotic polysaccharides may contain pentoses such as ribose or xylose, which are less common in prokaryotic ones. In addition to carbohydrates, EPSs contain several ester-linked substituents, such as acetate, succinate, and pyruvate ketals (Sutherland, 1990). Inorganic substituents, such as phosphate and sulfate, have also been widely reported. Phosphate is much more common and is found in EPSs from gram-negative bacteria (Sutherland, 1990). Sulfate appears especially in eukaryotic polysaccharides (Sutherland, 1990) and in EPSs from halophilic bacteria (Antón et al., 1988; Quesada et al., 2004; Mata et al., 2008; Llamas et al., 2010) and archaea (Parolís et al., 1996; Nicolaus et al., 2004). The succinate and pyruvate substituents, together with uronic acids, and phosphate and sulfate residues, contribute to the overall anionic charge of the polymers (Sutherland, 2001b). Anionic EPSs are normally linked to several cations. Thus, some alginates bind the divalent cations calcium, barium, and strontium (Sutherland, 1990), whereas EPSs from halophilic microorganisms link sodium and calcium (Amjres, personal communication).

EPSs are structurally classified into two main types: homopolysaccharides, which possess only one type of monosaccharide component, and heteropolysaccharides, which contain two or more types of monomer. Heteropolysaccharides have many applications in industry, the most widely used being xanthan, gellan, alginate, succinoglycan, and hyaluronic acid. Xanthan is secreted by bacteria of the genus *Xanthomonas* and is composed of glucose, mannose, glucuronic acid, and pyruvate and acetyl residues (Sutherland, 1990). Gellan and sphingon are related polymers produced by *Sphingomonas* species and are composed of tetrasaccharide repetitive units (Giavasis et al., 2000). Alginate is a linear polysaccharide composed of uronic acids and is produced by different algal and bacterial species (Clementi, 1997). Succinoglycan, a polymer of glucose and galactose with a tetrasaccharide branched chain, is produced by different soil bacteria (Simsek et al., 2009). Finally, hyaluronic acid is a linear polymer composed of glucuronic acid and *N*-acetylglucosamine and is produced by various different bacterial taxa (Sutherland, 1990).

14.3. PHYSICAL PROPERTIES OF EPSs

The physical properties of any microbial EPS depend on its chemical composition, structure, and molecular mass, all of which contribute to determining its final configuration. Polysaccharides are very long molecules with a molecular mass of around 0.5 to 2.0×10^6 Da

and can form fine strands (primary structure), double or triple helices (secondary structure), or a more complex network (tertiary structure). The linkages (1,2)- α and (1,6)- α give more flexibility to the strands and greater water solubility than (1,3)- α or (1,4)- α (Sutherland, 2001a). A low acyl content or low molecular mass results in polymers with low viscosity. Secondary structures are affected by organic and inorganic substituents and the presence of side chains (Sutherland, 1997, 2001b,c). In the tertiary structure the most favorable conformation includes charged peripheral macromolecules, which interact with water and with counterions to yield networks of highly viscous macromolecules, as occurs with xanthan, or even jellification, as occurs with alginate or gellan (Sutherland, 1990). These tertiary structures are usually found at low temperatures and in the presence of salts.

14.4. BIOLOGICAL FUNCTIONS OF EPSs

The production of EPSs involves a significant expenditure of carbon and energy by microorganisms, an expenditure that must afford benefits to the EPS producers as well as to those organisms associated with them. EPSs act as an adhesin and favor interactions and cellular associations among microorganisms, creating microenvironments within which the transfer of genes and metabolites is very common. Another important role is the protective function they provide against adverse physical and chemical factors or against attack by viruses and protists. They protect the microorganisms from extreme pH values, high temperatures, drying, freezing, biocides, detergents, and heavy-metal ions. Moreover, the production of EPSs is one way in which microorganisms can ensure their survival in nutrient-starved environments (Wolfaardt et al., 1999; Sutherland, 2001a).

14.5. EXOPOLYSACCHARIDES DERIVING FROM EXTREMOPHILIC ORGANISMS

Extreme environments have been identified as an important source of bacteria, archaea, algae, and fungi with interesting applications. Different organisms have developed different strategies to cope with adverse living conditions and the production of EPSs is a frequent survival strategy. For example, bacteria living in extreme marine environments such as those found in the cold waters of polar regions, in ocean trenches, or in deep-sea hydrothermal vents often use EPSs as an efficient protective barrier (Nichols et al., 2005). The protection conferred by EPSs in these hostile environments is achieved by the formation of biofilms to withstand high pressure and/or temperature, or by decreasing the freezing point of water in the vicinity of the bacteria (Nichols et al., 2005). Similar strategies are used by thermophilic bacteria found in terrestrial habitats (Lin et al., 2011). Nevertheless, EPSs are by no means the only products of extremophiles with potential applications. It has, in fact, been suggested that extremolytes, organic osmolytes produced by extremophile microorganisms, may have versatile applications in such diverse fields as cosmetics and medicine (Lentzen and Schwarz, 2006).

14.6. CLINICAL APPLICATIONS OF EPSs

During the last decade, EPSs obtained from different organisms have been discovered to harbor many hitherto unsuspected biomedical properties. One of the first bacterial EPSs

identified as having biomedical activity is produced by a strain of *Staphylococcus* and inhibits lymphocyte proliferation by activating the production of monocyte prostaglandins (Stout et al., 1992). Some fungal EPSs target prostate (Collins et al., 2006; Zhu et al., 2007) or lung (Guo et al., 2007) tumor cells by causing a cytotoxic effect that often involves either the triggering of or sensitization to death by apoptosis. Other fungal EPSs have been shown in vitro to exert insulinotropic activity, stimulating the production of insulin and protecting insulinoma cells from diabetogenic agents (Hwang et al., 2008). Finally, marine algae are also a valuable source of EPSs with potential biomedical applications. For example, treatment with porphyran, a sulfated EPS produced by *Porphyra*, triggers death by apoptosis in gastric tumor cells (Kwon and Nam, 2006, 2007).

Within this context of growing interest in uncovering the full array of biomedical applications of EPSs, the scrutiny of organisms living in extreme environments is an emerging field. Thus, an EPS produced by *Alteromonas infernus*, a bacterium isolated from a 2000-m-deep hydrothermal vent located in a Guaymas basin rift (Raguenes et al., 1997), is of particular interest in that an oversulfated modification exerts in vitro weak anticoagulant (Collicet et al., 2001) but strong angiogenic activity (Matou et al., 2005), which could be applied to accelerate vascular wound healing or to promote the growth of blood vessels in ischemic tissues. This oversulfated EPS also exerts effects on bone biology by inhibiting osteoclastogenesis, which leads to different levels of bone resorption regulation (Velasco et al., 2011).

Microorganisms that either tolerate or thrive in high-temperature environments produce EPSs with potential biomedical applications. *Bacillus licheniformis*, a thermotolerant bacterium isolated from a hot marine spring on Vulcano Island, Italy, produces two EPSs (EPS-1 and EPS-2) that block HSV-2 replication by enhancing the production of proinflammatory cytokines, particularly IL-12, IFN- γ , TNF- α , and IL-18 (Arena et al., 2006, 2009). This pattern of cytokine expression is compatible with the polarization of T-helper cells toward the Th1 compartment, thereby blocking viral replication, which opens up the possibility of using these EPSs to counter viral infections.

Another example of EPSs with immunomodulatory activity is found in one such polysaccharide deriving from the biofilm of the thermophilic bacterium *Thermus aquaticus* YT-1 that is able to exert a strong modulatory effect upon innate immunity by delivering signals through the Toll-like receptor 2 (TLR-2). Coupling of this receptor results in the stimulation of macrophages via the MyD88/TRAP pathway to recruit NF κ B, which ultimately leads to the secretion of cytokines, especially TNF- α and IL-6, as well as nitric oxide. This activation of macrophages and creation of an oxidative environment not only facilitates the killing of bacteria but also enhances antigen presentation by macrophages to T-cells (Lin et al., 2011). It has therefore been speculated that the EPS TA-1 could be used as a potent adjuvant because of its role as a TLR2 agonist (Lin et al., 2011).

The majority of EPSs with biological properties share the presence of sulfate groups in their composition, and evidence suggests that this is a critical feature that confers biological properties upon them (Wu and Chen, 2006). For example, the potent biomedical activities of both EPS-1 and EPS-2 produced by *Alteromonas infernus* are achieved only after chemical oversulfation, since the native polymers are biologically inert (Collicet et al., 2001). Other sulfated EPSs have many clinical applications as anticoagulant and antithrombotic (Ciancia et al., 2010), antiatherosclerotic (Engelberg, 1991), antiproliferative (Logeart et al., 1997) antiangiogenic (Ganesan et al., 2010), antimetastatic (Parish et al., 1987), anti-inflammatory (Matsui et al., 2003), complement-inhibiting (Blondin et al., 1994), and antiviral (Ghosh et al., 2009) agents.

14.7. EXOPOLYSACCHARIDES OF HALOPHILIC MICROORGANISMS

Halophilic microorganisms are types of extremophile adapted to living in saline and hypersaline habitats, where, in addition to high salt concentrations, other stress conditions, such as high solar radiation, alkaline pH values, and high temperatures, may prevail.

Noteworthy among the EPS-producing halophilic bacteria are 11 species of the family *Halomonadaceae* (Quesada et al., 1990; Bouchotroch et al., 2001; Martínez-Cánovas et al., 2004a; Martínez-Checa et al., 2005b; Gonzalez-Domenech et al., 2008a,b, 2009; Llamas et al., 2011), three species of the family *Alteromonadaceae* (Martínez-Checa et al., 2005a), the type species of the genera *Salipiger* and *Palleronia* (Martínez-Cánovas et al., 2004b; Martínez-Checa et al., 2005c), and the halophilic cyanobacterium *Aphanotece halophytica* (Oren, 2010). Among these polymers, the following are outstanding: mauran, produced by *Halomonas maura*, which, in a manner similar to xanthan, produces highly viscous aqueous solutions (Bouchotroch et al., 2000; Arias et al., 2003; Quesada et al., 2004); the polymers produced by *Halomonas eurihalina*, which originate gels at acid pH and have high emulsifying capacity (Quesada et al., 1993; 2004; Calvo et al., 1995, 1998; Béjar et al., 1996, 1998); the EPSs produced by *Halomonas ventosae*, *Halomonas anticariensis*, *Idiomarina fontislapidosi*, *Idiomarina ramblicola*, and *Alteromonas hispanica*, which have emulsifying properties (Mata et al., 2006, 2008); and the EPS produced by *Salipiger mucescens*, which contains a substantial quantity of fucose, a monosaccharide of great industrial interest in the fields of cosmetics and foodstuffs (Llamas et al., 2010). All these EPSs produced by halophilic bacteria have in common a high sulfate content (Arias et al., 2003; Quesada et al., 2004; Mata et al., 2006, 2008; Llamas et al., 2011), which is unusual in EPSs from prokaryotes, with the exception of those from the halophilic archaea *Haloferax mediterranei* and *Haloarcula japonica* (Antón et al., 1988; Parolis et al., 1996; Nicolaus et al., 2004).

14.7.1. *Halomonas stenophila* B-100

Because a distinct feature of many of these newly identified halophilic bacteria is the excretion of sulfated EPS, a considerable number of them have been further screened for biological activity. As a result it has been found that EPSs excreted by two newly discovered strains of *Halomonas stenophila* (B100 and N12) (Llamas et al., 2011) block the growth of human T-lymphocyte tumors (Ruiz-Ruiz et al., 2011). Strains B100 and N12 were originally isolated from soil samples taken from the Sabinar (B100) and San Pedro del Pinatar (N12) saline wetlands in Murcia, Spain.

It was found that a chemical modification yielded an oversulfated EPS that had significantly greater capacity to block T-cell tumor growth. This was not surprising since sulfate modifications have been shown to be a critical requirement in conferring biological activity upon EPSs (Collicec et al., 2001; Wu and Chen, 2006). An examination of the mechanisms of activity leading to tumor inhibition revealed that N12 merely induced cell arrest, whereas B100 was able to trigger massive cell death by apoptosis. Interestingly, this effect was restricted to the oversulfated polymer (B100S). Neither the native EPS B100 nor the native or oversulfated EPS N12 showed any cytotoxic effects despite their significant capacity to inhibit the growth of leukemic T-cells, thereby stressing the critical role that sulfate groups play in turning biologically inert EPSs into active compounds. An analysis of a large panel of primary and tumor cells of different origins demonstrated that the EPS B100S

selectively induced apoptosis in acute human T-lymphocyte leukemias (T-ALL). Tumor cells from other hematopoietic lineages or primary nontumor T-cells were not killed by B100S. Remarkably, we found that this oversulfated polymer also induced apoptosis in cells freshly isolated from the peripheral blood of patients with T-cell leukemias (Ruiz-Ruiz et al., 2011). The survival rate of T-ALL patients is today very unsatisfactory in adult patients, despite recent improvements in the applied treatments that have raised the five-year event-free survival in children under 5 years of age (Seibel, 2008). The potent cytotoxic activity of this EPS, together with the fact that it selectively targets leukemic cells, while skipping nontransformed populations, raises the attractive possibility of using EPSs as antitumoral agents.

A detailed biochemical analysis revealed that cell death was mediated by caspase activation (Ruiz-Ruiz et al., 2011). Furthermore, it was found that EPS B100S induced death by activating the intrinsic apoptotic pathway, as demonstrated by the kinetics of caspase activation, death protection by Bcl-2 and Bcl-xL overexpression, production of oxygen-reactive species, mitochondrial membrane potential changes, and Bak activation (Carranza et al., unpublished observations). A gene expression microarray revealed the critical role of calcium-dependent channels in the induction of B100S-mediated death by apoptosis (Carranza et al., unpublished data).

14.8. CONCLUDING REMARKS

Characterization of the biological properties of EPSs has emerged as an exciting field due to the wide array of biological properties discovered to date. In addition to the physical properties that have turned out to be useful for industrial applications, some EPSs produced by extremophile organisms are currently attracting considerable attention because of their antitumoral and immunomodulatory properties, among others.

Within this context, an EPS excreted by the newly discovered halophilic bacterium *Halomonas stenophila* B100 has been identified, which upon chemical oversulfation is turned into a potent antitumoral agent that selectively targets T-ALL cells. The unsuspected and interesting biological properties of B100S and other EPSs produced by bacteria isolated from hostile habitats suggest the need for further efforts to isolate and screen new extremophile bacteria, the products of which might be turned into biologically active agents.

Acknowledgments

We are grateful to our colleague Dr. J. Trout for helpful suggestions and improving the text.

Our work has been supported by grants P07-CVI-3150 from the Department of Innovation and Science, Regional Government of Andalucia (to E. Quesada), SAS111218 from the Andalusian Health Service, and PI10/01567 from the Health Research Fund, Ministry of Science and Innovation of Spain (to I. J. Molina).

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BIOSYNTHESIS OF EXTREMOLYTES: RADIATION RESISTANCE AND BIOTECHNOLOGICAL IMPLICATIONS

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15.1. INTRODUCTION

Driven by increasing industrial demands for biocatalysts, enzymes, and metabolites that can cope with industrial process conditions, considerable efforts have been made to search for such products. As a result, the characterization of microorganisms that are able to thrive in extreme environments has received a great deal of attention (Van den Burg, 2003). These organisms, termed *extremophiles*, are a valuable source of enzymes and metabolites used in industrial processes. The extreme conditions in which they live can be physical extremes (e.g., temperature, pressure, radiation) or chemical conditions such as salinity and pH (Raspor and Zupan, 2006; Oarga, 2009). Radiation-resistant microorganisms are known to survive in ionizing and nonionizing radiation environments that could be lethal to others (Rainey et al., 2005; Singh and Gabani, 2011). The knowledge that extremophiles are capable of surviving under nonstandard conditions has led to the assumption that the properties of their metabolic products and enzymes have been optimized for these conditions. However, the biomolecules (e.g., cryoprotectants, antifreeze proteins, membrane stabilizer lipids, antioxidants, antiradiation agents, various other small molecules of therapeutic value) could also result from the extremophilic origin. As a result, it has been hypothesized that biomolecules obtained from these microorganisms could be applicable in similarly diverse conditions.

TABLE 15.1. Potential Industrial Applications of Enzymes Isolated from Extremophiles

Enzyme Category	Industrial Applications	Extremophiles
Proteases	Baking, brewing, detergents	Thermophile
Amylases	Glucose and fructose for sweeteners	
DNA polymerase	Genetic engineering	
Xylanases	Paper bleaching	
Amylases	Polymer degradation in detergents	Psychrophile
Proteases	Cheese maturation, dairy production	
Dehydrogenases	Biosensors	
Cellulases	Polymer degradation in detergents	Alkalophile
Sulfur oxidation	Desulfurization of coal	Acidophile
Chalcopyrite concentrate	Valuable metals recovery	
Microorganisms	Fermentation of poly(γ -glutamic acid) and poly(β -hydroxybuty acid)	Halophiles
	Starch granule and gel formation	Piezophiles

To this day, several types of extremophiles have been identified (Kato and Takai, 2000; Irwin and Baird, 2004). Thermophilic extremophiles, or *thermophiles*, have been known to live at extreme temperatures ($>50^{\circ}\text{C}$). Since many reactions involved in industrial processes occur in high-temperature water-based solutions, thermophilic extremozymes have attracted much attention (Egorova and Antranikian, 2005). Many reactions also take place at high salt and ion concentrations that have been known to denature proteins; enzymes from halophiles, extremophiles that are able to survive in hypersaline habitats (Madern et al., 2004; Sabet et al., 2009), can be used to accomplish such reactions. Extremozymes from psychrophiles, able to survive at low temperatures ($<20^{\circ}\text{C}$) (D'Amico et al., 2006; Cavicchioli et al., 2011), have also been investigated for industrial processes due to efforts to decrease energy consumption. With enzymes able to function at low temperatures, it is feasible for manufacturing plants to run processes at lower temperatures, resulting in decreased energy consumption and lower costs for the production of goods. There has been extensive research on the structural proteins and key metabolic enzymes that are responsible for the organisms' unusual properties. Table 15.1 summarizes some of the interesting enzyme classes for use in industrial biotransformations that are active or stable under extreme conditions.

The evolutionary relationship of most of the extremophiles with their present-day unfavorable environments indicates novel genomic pools, biomolecules, and metabolic uniqueness of these microorganisms (Schiraldi and De Rosa, 2002). In the modern era, discovery of the extremophiles has sensitized the biotechnology industries and entrepreneur houses to develop novel, unconventional products (Kumar et al., 2011). Additional types of extremophiles include metallophiles, which grow in the presence of high metal concentrations; radiophiles, which grow in the presence of high radiation; and microaerophiles, which grow under oxygen deprivation (Van den Burg, 2003). The biotechnological applications of enzymes from such extremophiles are always the center of interest. Therefore, in view of the tremendous industrial and therapeutic potential and future applications of extremophiles, the current chapter sheds light on particular groups of extremophiles, the majority of which possess radiation resistance. We also provide insight on the microbial biosynthesis of extremolytes and extremozymes.

15.2. BIOTECHNOLOGICAL IMPLICATIONS OF EXTREMOLYTES

15.2.1. Industrial Implications

The unique features of the wide variety of extremophiles have allowed for far-reaching applications in biotechnology, ranging from bioremediation of toxic compounds to the production of medically important drugs (Gomes and Steiner, 2004; Kumar et al., 2011). There are several advantages to conducting reactions at extreme conditions: for example, elevated temperatures allow for increased solubility of many polymeric substrates and faster reaction rates. Enzymes derived from extremophiles, or *extremozymes*, are considered promising for such reactions, due to their high stability when exposed to extreme heat, pressure, pH, salinity, and radiation (Table 15.1).

Many extremozymes involved in carbohydrate metabolism are of great industrial interest. The starch-processing industry converts starch into more valuable products such as dextrins, glucose, fructose, and trehalose. In all starch-converting processes, high temperatures ($>50^{\circ}\text{C}$) are necessary to melt starch and make it available to enzymes. However, at these temperatures ($>50^{\circ}\text{C}$), most enzymes become inactive to process enzymatic reactions (Moradian and Benner, 1992). Thermostable amylases extracted from thermophiles are potentially usable in such reactions to lower the cost of products made from starch. Satyanarayana et al. (2004) have demonstrated that amylase isolated from thermophiles exhibits optimal activity at 100°C . Thermostable and thermoactive pullulanases, which hydrolyze branched oligosaccharides, have been shown to be optimal between temperatures of 90 and 105°C (Kriegshauser and Liebl, 2000). These extremophilic debranching enzymes are promising candidates to optimize starch conversion by running the process at higher temperatures. Purified glucoamylases and β -glucosidases from thermophiles have been known to produce β -D-glucose from nonreducing ends of polysaccharides, providing the potential to improve industrial glucose production (Hye-Jung et al., 2009). The α - and β -glucosidases are best characterized for conversion of smaller oligosaccharides that have α -anomeric configuration (Antranikian et al., 2005). The use of thermostable enzymes in starch processing will allow for a cheaper and faster process with a higher yield of products (Fig. 15.1A).

Cellulose is the most abundant organic polymer that can be hydrolyzed to glucose via endoglucanase, exoglucanase, and β -glucosidase (Schiraldi et al., 2002; Morais et al., 2010). Many β -glucosidases were found in thermophiles that showed optimal activity at 103°C (Antranikian et al., 2005). Thermostable endoglucanases are able to degrade β -(1,4) linkages in cellulose and crystalline cellulose, which is a major component of plant biomass (Graham et al., 2011). These enzymes, along with amylases, can be used to produce glucose from cellulose. There is a great demand for such cellulases in various industrial applications, including biofuel production (Fig. 15.1B). The commercial interest in the applications of xylanases, which degrade hemicelluloses, has grown significantly (Saleem et al., 2002). Xylanases are able to attack xylans (plant cell wall fraction) without affecting cellulose, and as a result have shown some potential for biobleaching of pulp and paper (Subramaniam and Prema, 2002). Also, the conversion of xylan to xylose has been shown to ferment into ethanol (Fig. 15.1C) (Lee et al., 2010). As most of these reactions in the industries occur at extreme temperatures, it has been theorized that xylanases from extremophiles may be useful in catalyzing the reactions.

As a method for detection of biological molecules at the molecular level, Polymerase chain reaction (PCR) has revolutionized the field of molecular biology during the past

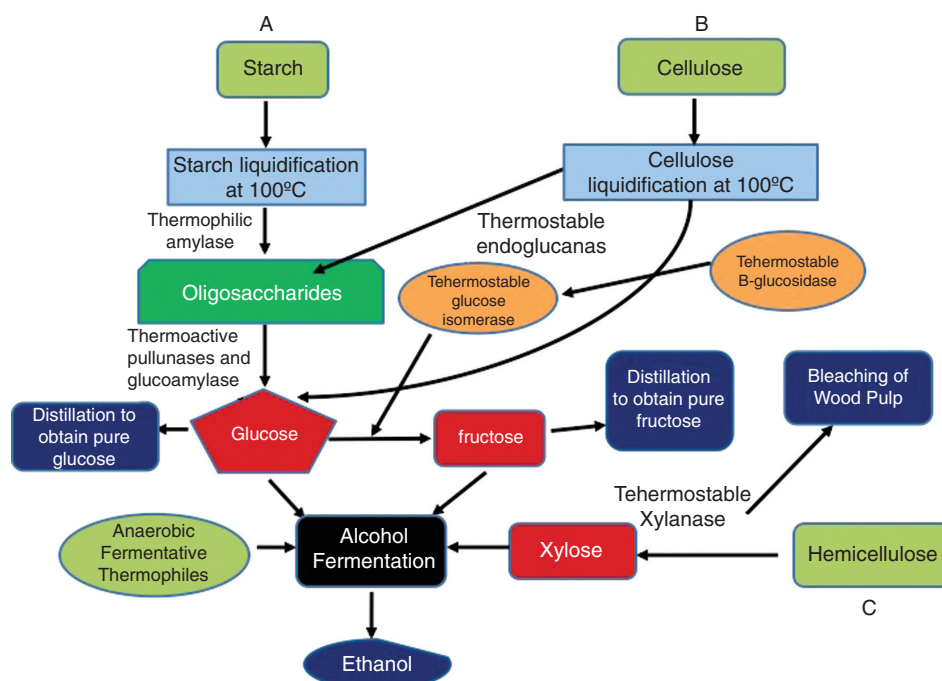


Figure 15.1. Extremozymes mediated production of sugars and ethanol from unconventional carbon sources found in thermophiles. (A) Conversion of starch; (B) conversion of cellulose; (C) conversion of hemicellulose. (See insert for color representation of the figure.)

20 years (Mullis et al., 1986). Thermostable DNA polymerase plays a major role in this process; extremophilic polymerase has shown low error rates, high processivity, and high extension rates, which result in accurate amplification of target DNA sequences (Hashimoto et al., 2001). DNA polymerase and DNA ligase from extremophiles have great promise for the improvement of molecular biology in the near future.

In another application, ester hydrolases have been identified in all domains of life. They are able to catalyze hydrolysis of an ester bond, which results in the formation of an alcohol, usually optically pure, and carboxylic acid. In addition, they can catalyze the reverse reaction, known as *transesterification* (Krishna and Karanth, 2002). A need for extremozymes has been recorded in detergent production, organic synthesis, biodiesel production, flavor and aroma synthesis, and other food-related processes (Panda and Gowrishankar, 2005; Salameh and Wiegel, 2007). Since most industrial processes occur at elevated temperatures, ester hydrolases from thermophiles could be especially useful in these processes.

15.2.2. Therapeutic Implications

Radiation-Resistant Extremophiles. In addition to their industrial uses, secondary metabolites from extremophiles have been recognized as having multiple therapeutic applications. Radiation protection is one of the most important of these.

The skin is the largest organ of the human body and the primary target for an array of environmental factors; protection of skin from every type of radiation, including nuclear and rays, is inevitable. Ultraviolet radiation (UVR) from the sun reaches the Earth's surface in the form of electromagnetic radiation with a wavelength of 10 to 400 nm and energies ranging from 3 to 124 eV. This ionizing radiation causes changes in molecular structures and the formation of free radicals. UVR has been linked to many harmful skin effects, including immune suppression, dermatitis, premature aging, and skin cancer (Hockberger, 2002; Agar et al., 2004; Kumar et al., 2010; Singh and Gabani, 2011). Melanin can prevent damage to skin, but increased exposure to UVR can deplete its defenses and leave the skin vulnerable to attack from reactive oxygen species (ROS).

In one study, desiccation-tolerant cells of *Chroococcidiopsis* sp. CCMEE029 exhibited reduced ROS accumulation and dehydrogenase activity upon rewetting (Billi, 2009), indicating that their microbial metabolic products could be of interest for producing cosmetic treatments for human skin suffering from extreme dryness. In another, gamma- and UVR-resistant strains of *Deinococcus depolymerans* were isolated from radioactive sites in Japan (Asker et al., 2011). These strains contained red pigments that were hypothesized to contribute to microbial resistance against high-energy radiation.

Certain other extremolytes from extremophiles have been explored for therapeutic implications, including mycosporine-like amino acids (MAAs) (Singh et al., 2010), scytonemin (Stevenson et al., 2002a,b), bacterioruberin (Asgaranai et al., 2000), and ectoine (Bünger and Driller, 2004). These extremolytes could potentially be incorporated into cosmetics to block harmful effects of UV radiation, and a variety of additional applications are shown in Table 15.2.

Scytonemin has been known to act as a sunscreen by blocking UVA radiation, and therapeutically has antiproliferative and anti-inflammatory properties (Stevenson et al., 2002b). UV radiation induces the scytonemin gene cluster (Syc), which produces scytonemin to block the incoming UV radiation (Fig. 15.2A). In addition to this passive activity, scytonemin has also been known to inhibit PLK1, which is theorized to control oncogenes (Fig. 15.2B) (Stevenson et al., 2002b). A recent study showed that cells in which mutant Ras acts as an oncogene are highly sensitive to PLK1 inhibition, because mitotic stress seems to be a key event in these cells (Luo et al., 2009). It is hypothesized that scytonemin can be used to inhibit PLK1 to induce apoptosis in cancer cells.

In addition to scytonemin, halophilic microorganisms synthesize ectoine, which has been known to protect the skin from the harmful effects of UV radiation. It has been shown that UV-radiation-induced second messenger release, transcription factor AP-2 activation, intercellular adhesion molecule-1 expression, and mitochondrial DNA mutation can be prevented by ectoine (Fig. 15.3) (Bünger and Driller, 2004).

The industrial uses of extremolytes are not well known; however, the integration of these metabolites into cosmetic products has potential for protecting skin from UV-induced damage.

15.3. FERMENTATIVE PRODUCTION OF EXTREMOLYTES

15.3.1. Microorganisms: An Asset in Extremolyte Fermentation

Microorganisms have excelled at producing primary and secondary metabolites from a variety of raw carbohydrates for billions of years. In recent years, the extremophiles have

TABLE 15.2. Potential Therapeutic Applications of Various Extremolytes

Therapeutic Targets/Implications	Extremolyte	Microorganisms/ Source	References
<i>Protection of Biological Macromolecules</i>			
Stabilization of enzymes against thermal stress and freeze drying	Mannosylglycerate	<i>P. furiosus</i> , <i>P. mendocina</i> , and <i>R. marinus</i>	Borges et al., 2002
Stabilization of recombinant nuclease	Mannosylglycerate	<i>Staphylococcus aureus</i>	Faria et al., 2004
Thermostabilization of rubredoxin	DGP	<i>Desulfovibrio gigas</i>	Lamosa et al., 2003
Inhibition of insulin amyloid formation	Ectoine	<i>Pyrococcus aerophilum</i> and <i>Thermus thermophilus</i>	Arora et al., 2004; Cruz et al., 2006
Stabilization of retroviral vaccines	Mannosylglycerate; hydroxyectoine	Virus	
Reduction of apoptotic cell death induced by MJD gene product	Ectoine	Machado–Joseph disease gene	Furusho et al., 2005
Inhibition of aggregation and neurotoxicity of Alzheimer's β -amyloid	Ectoine, hydroxyectoine	Alzheimer's β -amyloid	Kanapathipillai et al., 2005
Kinase inhibitory activity	Scytonemin	Cyanobacteria	Stevenson et al., 2002a,b
<i>Protection of Cells</i>			
Stabilization of <i>E. coli</i> during drying and storage	Ectoine; hydroxyectoine	<i>Escherichia coli</i>	Manzanera et al., 2004
Block of UVA-induced ceramide release in human keratinocytes	Ectoine	Human keratinocytes	Grether-Beck et al., 2005
Protection of mitochondrial DNA in human dermal fibroblasts	Ectoine	Human keratinocytes	Bünger and Driller, 2004
Antioxidant activity	Usurijene	<i>Synechocystis</i> sp.	Zhang et al., 2007
Antioxidant ability	Palythenic acid	<i>Maristentor dinoferus</i>	Lobban et al., 2002
<i>Protection of Skin</i>			
Prevention of UVA-induced photoaging	Ectoine	Human keratinocytes	Bünger and Driller, 2004
Cytoprotection of keratinocytes	Ectoine	Human keratinocytes	Buommino et al., 2005
Skin, sunscreen	Mycosporine- taurine	<i>Heterocapsa</i> sp.	Montero and Lubian, 2003
UV absorption, sunscreen	Palythine	<i>Porphyra umbilicalis</i>	Karsten et al., 2009

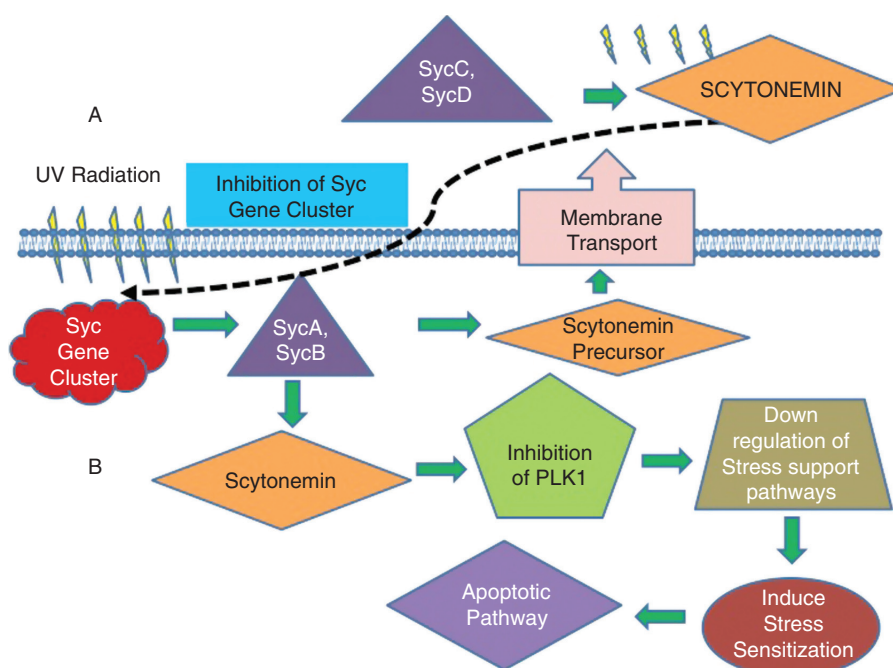


Figure 15.2. Escape mechanism from ultraviolet (UV) radiation led to therapeutics. (A) UVA is absorbed and induces the Syc gene cluster to produce scytonemin. Scytonemin is transported outside the cell membrane, where its accumulation ultimately blocks UV radiation. (B) The inhibition of PLK1 is hypothesized to induce stress sensitization by downregulating the activity of stress support pathways, leading ultimately to apoptosis of cancer cells. (See insert for color representation of the figure.)

been considered as selective microorganisms to create value-added products of commercial interest (Turner et al., 2007). In nature, the best-known hyperthermophilic microorganisms are archaeobacteria. The most suitable example of a thermophilic archaeobacterium is *Pyrolobus fumarii* (Crenarchaeota), a nitrate-reducing chemolithoautotrophic bacterium, grown at 113°C (Blöchl et al., 1997). Surprisingly, some enzymes derived from hyperthermophilic bacteria have shown maximum catalytic activity at unusually high temperatures: for example, in one study, maximum amylopullulanase activity was reported at 142°C (Schuliger et al., 1993). There are distinguished thermophiles among the phototrophic bacteria (cyanobacteria, purple and green bacteria), eubacteria (*Bacillus*, *Clostridium*, *Thiobacillus*, *Desulfotomaculum*, *Thermus*, lactic acid bacteria, actinomycetes, spirochetes, etc.), and archaea (*Pyrococcus*, *Thermococcus*, *Thermoplasma*, *Sulfolobus*, and other methanogens).

Radiation is energy in the form of either particles (i.e., neutrons, electrons, protons, alpha and beta particles, or heavy ions) or electromagnetic waves (i.e., γ -rays, x-rays, ultraviolet radiation, visible light, infrared, microwaves, radio waves, etc.). The effects of UV and ionizing radiation on living systems are well studied because of their importance in medicine, energy production, warfare, and space programs (Huang et al., 2007). In the last several decades of the twentieth century, numerous true extreme-loving organisms were

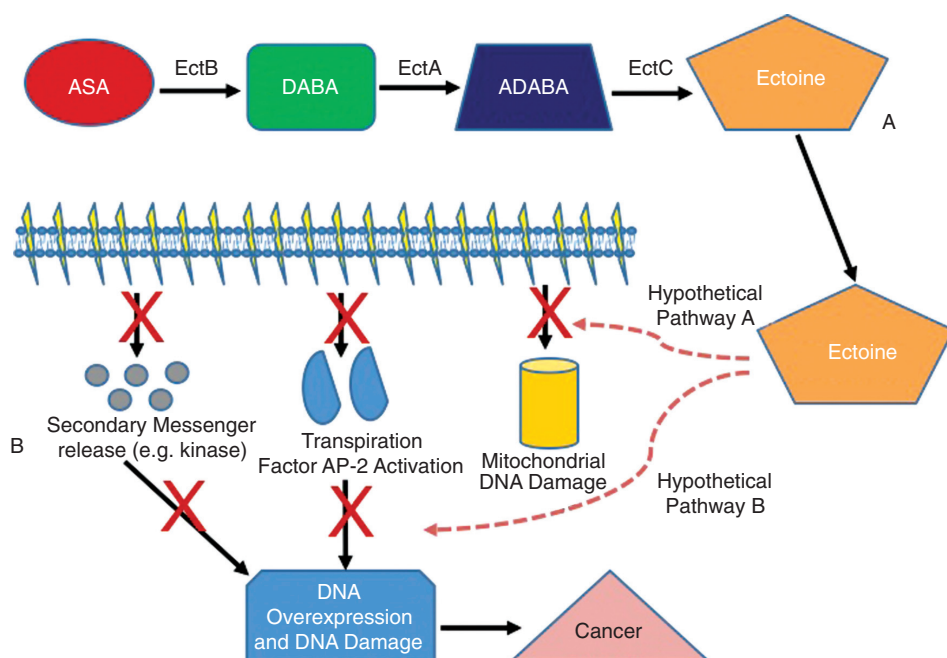


Figure 15.3. Biosynthesis of extremolytes in halophilic bacterium *H. elongate* and proposed hypothetical survival mechanism. (A) Biosynthesis of multiple ectoines in *H. elongate* (ASA, aspartate semialdehyde, DABA; 1-2,4-diaminobutyrate; ADABA, *N*-acetyl-1-2,4-diaminobutyrate). (B) Hypothetical pathway A: ectoine could act as a UV blocker and prevents the induction of secondary messengers, transcription factor AP-2 activation, and mitochondrial DNA mutations. Hypothetical pathway B: ectoine may also act as an inhibitor and blocks the secondary messengers and transcription factor AP-2 from binding to promoters and halts DNA transcription. (See insert for color representation of the figure.)

discovered, including *Deinococcus radiodurans*, the frontrunner among the radioresistant bacteria, which can tolerate 1000 times more gamma radiation than can normal microbial species (Makarova et al., 2001). Radiation background levels beyond the accepted limit are sufficient to qualify an organism for extremophile status.

D. radiodurans is famous for its ability to withstand supralethal ionizing radiation (up to 20 kGy of γ -radiation) and UV radiation (doses up to 1000 J/m²) (Battista, 1997). The extraordinary resistance of *D. radiodurans* is thought to be a by-product of its resistance to extreme desiccation. Other organisms that can stand high levels of radiation are *Rubrobacter* sp. (Ferreira et al., 1999) and the green alga *Dunaliella bardawil* (Ben-Amotz and Avron, 1990). The endolithic cyanobacteria protect themselves from UV through their natural habitats (porous rocks) and by producing photoprotective pigments. The desiccation-resistant strains of the cyanobacterium *Chroococcidiopsis* also exhibit resistance against ionizing radiation, probably due to efficient DNA repair mechanisms (Billi, 2009). Biosynthesis of radiation-responsive pigments and DNA repair enzymes via modern biotechnological techniques may allow other organisms to live in radiation-rich environments such as the Martian environment.

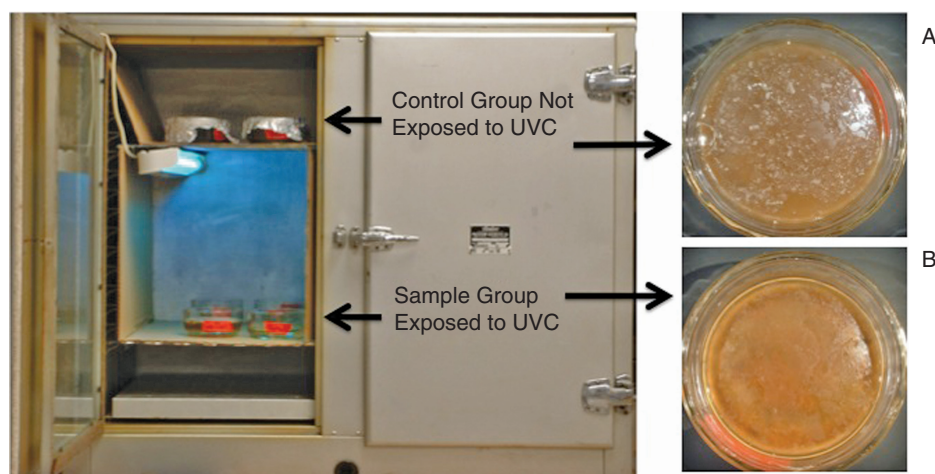


Figure 15.4. UV radiation chamber for isolation of UVR-resistant microorganisms. (A) Control group, not exposed to the UV, shows the cloudy appearance of microbial flora in nutrient broth medium. (B) Sample group, exposed to the UV, shows comparably fewer microorganisms on the surface of the medium after continuous UV exposure for 7 days in both groups at 37°C. (See insert for color representation of the figure.)

In the thrust to explore the characteristics of radiation-resistant microorganisms, we attempted to study microbial flora at elevated land (2245 ft) that demonstrated UVR resistance. Soil samples were collected during the month of August at Tracy Ridge recreation area in the Allegheny National Forest, located in northwestern Pennsylvania. The samples were enriched in nutrient broth (NB) and potato dextrose broth (PDB) medium under germicidal ultraviolet light subtype C (UVC) with a dose rate of 12.8 J/m^2 (intensity of 4.8 W) at 37°C in a home-designed UV chamber (Fig. 15.4). The rate of microbial survivability under UV and non-UV was monitored every 24 h for 192 h on nutrient agar plates. Visual observation clearly indicated an abundance of microbial growth in the non-UV bowl (Fig. 15.4a) compared with the UV-exposed bowl (Fig. 15.4b). The highest survival rates occurred at a UV dose of $2.22 \times 10^6 \text{ J/m}^2$ (48 h) (Fig. 15.5), compared to the non-UV-exposed medium (96 h). Further studies are aimed at identifying potential microorganisms using 16S rRNA sequencing, as well as their proteins and metabolites that were produced after UVR exposure, and predicting their therapeutic and commercial significance.

15.3.2. Evaluation of the Fermentation Process

Fermentation Medium

CHEMICAL PARAMETERS. Several nutrients are essential for fermentation reactions to occur. A variety of constituents have been defined for fermentation media that enable microorganisms to produce specific extremolytes, such as ectoine. Multiple studies have demonstrated that the concentrations of sodium chloride (NaCl) and monosodium glutamate (MSG) have a significant influence on cell growth and ectoine synthesis (Onraedt et al., 2005; Roberts, 2005; Saum and Müller, 2008; Van-Thuoc et al., 2010). By varying

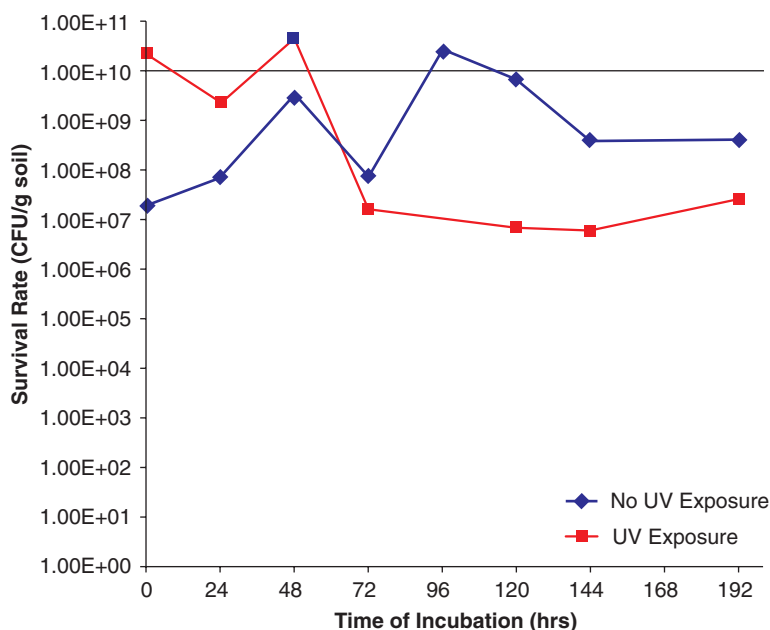


Figure 15.5. Survivability of UVR resistance in total microbial flora from higher elevation. The total microbial survivability was determined using serial dilution method on respective agar medium.

the concentrations of NaCl and glutamate, researchers have attempted to optimize the production of useful extremolytes (Guzmán et al., 2009; Zhang et al., 2009; Van-Thuoc et al., 2010).

Zhang et al. (2009) studied an ectoine-excreting halophile known as *Halomonas salina* using a medium that contained an inorganic salt mixture of NaCl, KH_2PO_4 , K_2HPO_4 , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$. It was found that although the extracellular concentration of ectoine was reduced as the NaCl concentration was increased, the intracellular accumulation of ectoine was increased, and therefore the total ectoine production was unaffected by the concentration of NaCl within the range 0.5 to 2.0 mM (29.2 to 116.8 g/L). However, the maximum growth rate and cell density occurred at a concentration of 0.5 mM NaCl (29.2 g/L). The relatively low NaCl concentration was not only beneficial for cell growth, but also reduced corrosion of the equipment. The concentrations of MSG, glucose, and yeast extract were also varied with the goal of optimization, and the maximum ectoine production was found to be 245.9 mg/g cell dry weight (CDW) at a concentration of 150 mM/L MSG. While the addition of glucose had no effect on ectoine synthesis, the addition of yeast extract was found to decrease the amount of ectoine production. The combination of optimized medium components led to a final ectoine concentration of 357.5 mg/g CDW, with a volumetric productivity of 7.9 g/L per day (Zhang et al., 2009).

Using a medium containing NaCl, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, NH_4Cl , K_2HPO_4 , and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, Guzmán et al. (2009) confirmed the favorable effects of low NaCl concentrations (5 to 7.5% w/v) on cell growth of *Halomonas boliviensis* and found that these concentrations also favored the accumulation of poly(3-hydroxybutyrate) (PHB). However, maximum ectoine concentration (0.74 g/L) was found to occur at a NaCl concentration of 10 to 15% w/v.

These findings led the group to work toward optimizing the coproduction of both ectoine and PHB. Production of ectoine was achieved using a two-step fed-batch process: the first step for maximizing biomass production and the second step for maximizing ectoine synthesis. In the first step, a 4.5% w/v NaCl concentration was used for 21 h when the CDW reached 10.7 g/L. In the second step, the cells were transferred to a fresh batch medium that contained 12.5% w/v NaCl. After 18 h of cultivation, the ectoine content reached a maximum of 17.0 wt% CDW (5.5 to 5.7 g/L), providing a total volumetric production of 3.4 g/L per day.

During the combined production of ectoine and PHB, the first fed-batch cultivation was modified to avoid any nitrogen limitation and further improve cell density. For this, MSG, NH_4Cl , and K_2HPO_4 were fed to the bioreactor to maintain their initial concentrations, resulting in a CDW of 11.7 g/L after 15 h. The second step was performed at a NaCl concentration of 7.5% w/v, and the glutamate, NH_4Cl , and K_2HPO_4 levels were maintained for the first 3 h, after which the supply was stopped and the consumption of nutrients was monitored. It was found that glutamate and NH_4Cl were consumed completely within 12 h, whereas the phosphate levels remained the same after a slight initial decrease. The cell density, PHB content, and ectoine concentration reached a maximum of 62 g/L, 68.5 wt%, and 4.3 g/L, respectively, at 24 h of cultivation, providing a volumetric productivity of 1.06 g/L per day for PHB and 2.8 g/L per day for ectoine. The results also indicated that an increase in cell mass led to an increase in the production of ectoine, whereas a constant cell mass led to the production of PHB (Guzmán et al., 2009).

Van-Thuoc et al. (2010) utilized *H. boliviensis* in their attempt to optimize biomass and ectoine production using response surface methodology and a medium containing an inorganic salt mixture of NaCl, NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, K_2HPO_4 , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, and Tris. A second-order polynomial model was created using MATLAB software and was used to optimize the medium used for biomass and ectoine production. The biomass concentrations were calculated for a total of 20 different combinations of NH_4Cl , $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and K_2HPO_4 concentrations ranging from 1.36 to 3.04 g/L, 1.65 to 4.35 g/L, and 0.53 to 1.87 g/L, respectively, and the various combinations were then performed experimentally. Among the three constituents, the phosphate salt had the most effect on biomass production, as K_2HPO_4 provides elements for biomass and the K^+ ions are accumulated intracellularly to maintain turgor pressure and cell volume. The ectoine concentration was calculated for 12 different combinations of NaCl and MSG concentrations ranging from 63.4 to 176.6 g/L and 2.93 to 17.07 g/L, respectively, and the combinations were then performed experimentally. The actual maximum biomass concentration was achieved (3.36 g/L) at a concentration of 2 g/L NH_4Cl , 3.47 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.77 g/L K_2HPO_4 , while the maximum ectoine concentration of 1.25 g/L was observed at a concentration of 157.3 g/L NaCl and 10.6 g/L MSG, resulting in an ectoine volumetric productivity of 6.3 g/L per day (Van-Thuoc et al., 2010). The results from these experiments, as well as the results from several other experiments, are summarized in Table 15.3.

Among refined carbohydrate sources, glucose was used as a carbon source for ectoine production in a two-step fed-batch culture of *H. boliviensis* (Guzmán et al., 2009). Zhang et al. (2009) utilized monosodium glutamate as the sole source of carbon and nitrogen in ectoine production, while Van-Thuoc et al. (2010) used a combination of glucose and monosodium glutamate in a two-step fed-batch culture of *H. boliviensis*.

Glutamate and NH_4^+ were used as the nitrogen sources and phosphate was used as the phosphorus source for ectoine production (Guzmán et al., 2009). For combined ectoine and

TABLE 15.3. Comparison of Various Studies for Optimization of Ectoine Production

Organism	NaCl (g/L)	MSG (g/L)	CDW (g/L)	Ectoine Concentration (g/L)	Volumetric Productivity (g/L · day)	References
<i>Halomonas salina</i> DSM 5928	29.2	253.7	19.4	6.9	7.9	Zhang et al., 2009
<i>Halomonas boliviensis</i>	125	20	10	5.7	3.4	Guzmán et al., 2009
	157.3	10.6	28.7	1.25	6.3	Van-Thuoc et al., 2010
<i>Halomonas elongata</i> ^a	150	0	48	7.4	5.3	Sauer and Galinski, 1998
<i>Brevibacterium</i> <i>epidermis</i>	58.4	50	49	8	2	Onraedt et al., 2005
<i>Halomonas salina</i> DSM 5928	150	0	N/A	0.41	N/A	Zhang et al., 2009
	58.4	50	N/A	1.509	N/A	Zhang et al., 2009
<i>Brevibacterium</i> <i>epidermis</i>	58.4	50	7	0.14	N/A	Onraedt et al., 2004

^aAfter nine cycles of bacterial milking.

PHB production, NH₄Cl and glutamine were used as the nitrogen sources, while K₂HPO₄ was used as the phosphorus source (Van-Thuoc et al., 2010).

PHYSICAL PARAMETERS pH is one of the most viable factors for continuing extremolyte fermentation. Fundamental investigations revealed that extremophiles were metabolically active when the pH of fermentation medium was adjusted to 7.5 using concentrated HCl or 5 M NaOH (Guzmán et al., 2009). A lower pH value may retard the rate of the fermentation reaction, which may be followed by deterioration in extremolyte accumulation. The pH was maintained at 7.2 by adding 5 M HCl for extracellular synthesis of ectoine (Zhang et al., 2009). During fed-batch fermentation, the pH was adjusted to 7.5 using 5 M HCl solution (Van-Thuoc et al., 2010). In the investigation by Guzmán et al. (2009), the average temperature during cultivation was maintained at 35°C, which is highly important for the production of extremozymes in order to liberate extremolytes of interest from a variety of microorganisms. In the other studies, the temperature was maintained at 30°C throughout the fermentation process (Zhang et al., 2009; Van-Thuoc et al., 2010).

Oxygen supply to the extremophiles is a key parameter. The stirring velocity was initially set to 700 rpm and the aeration rate was set to 1 L/min. When the initial oxygen concentration decreased by 80%, the stirring velocity and air inflow rate was increased to 1100 rpm and 5 L/min, respectively (Guzmán et al., 2009; Van-Thuoc et al., 2010). The shake-flask strains were cultivated on a rotary shaker set at 120 rpm (Zhang et al., 2009) to provide proper aeration.

Fermentation Types for Extremolyte Production.

BATCH AND FED-BATCH FERMENTATION. Efficient technologies are required to decrease the overall cost of extremolytes and extremozymes. Metabolically active extremophiles will maximize the efficiency of extremolyte production under suitable culture conditions. To

achieve the growth of viable (living) cells of extremophiles, a small quantity of cells has to be verified in liquid medium that contains a suitable amount of nutrients at the proper pH and temperature. To develop a successful batch fermentation process, the kinetic description must first be considered. The biological reactors (or bioreactors) used to optimize and control the bioprocesses involve two or more phases: a single gas phase, at least one liquid phase, and at least one solid phase. The cells of interest are typically in contact with a liquid phase and are either suspended in the liquid, as in a suspension culture, or attached to a suitable solid support, as in an immobilized culture. The interactions between the cells (or the biotic phase) and the liquid (or the abiotic phase) must be considered and accounted for completely during development of the fermentation method. The abiotic phase contains the essential nutrients for the cells, as well as end products resulting from cellular metabolism, which are then excreted into the medium. A slight variation in pH could greatly influence the cellular activities and transportation of extremolytes; therefore, the pH of the bioreactor must be optimized and controlled throughout the fermentation process. To ensure that there is no oxygen depletion, the mixtures are shaken or aerated during cultivation (Cinar et al., 2003).

During batch cultivation, the essential nutrients being added to the microbial cultivation may limit the nutrient supply, inhibiting cellular growth, which results in low final cell counts and product concentration. Once the cells reach the stationary growth phase, they are removed and a new batch of cells undergoes the fermentation process. To overcome this nutrient limitation, fed-batch cultivation has been adapted widely and is currently used most often for cell culture processes. During the traditional fed-batch fermentation, concentrated feed medium is added once the nutrient levels start to become depleted, prolonging the growth phase and increasing the final cell count and product concentration. In this process, the medium is added proportionally to cell concentration, either continuously or intermittently. During intermittent-harvest fermentation, a portion of the cells and product are removed during the stationary growth phase and replenished with fresh medium. This process is repeated several times, allowing for an extended production period (Wlaschin and Hu, 2006).

In both batch and fed-batch processes, lactate ammonium and other metabolites eventually accumulate in the culture broth. These and other factors, such as high osmolality and accumulation of reactive oxygen species, have been shown to inhibit cell growth, productivity, and metabolism (Ozturk et al., 1992; Wlaschin and Hu, 2006). To extend the duration of batch and fed-batch cultivation processes, the metabolite accumulation must be minimized. Typically, this reduction in metabolite accumulation is achieved by limiting the availability of glucose and glutamine, using certain feeding strategies. This directs the cell metabolism to a more efficient state and decreases the amount of lactate and ammonium production, which is commonly known as a *metabolic shift*.

The large-scale production of ectoines is achieved using a process known as *bacterial milking* (Sauer and Galinski, 1998; Guzmán et al., 2009). In this process, the halophilic bacterium *Halomonas elongata* is cultivated using fed-batch fermentation at 15 to 20% w/v NaCl. *H. elongata* accumulates ectoines intracellularly during the fermentation process and is then subjected to osmotic down-shock, which activates mechanosensitive channels, producing extracellular ectoines (Sauer and Galinski, 1998; Lentzen and Schwarz, 2006). The cells are then subcultured; 7.4 g/L ectoine production was observed after nine cycles of the bacterial milking process, with a volumetric productivity of 5.3 g/L per day (Sauer and Galinski, 1998; Guzmán et al., 2009).

CONTINUOUS FERMENTATION. The continuous fermentation process was investigated to avoid the interruptions inherent in the batch and fed-batch fermentation processes. In the continuous fermentation process, the cell and broth mixture is centrifuged after fermentation has taken place and separated into three components: fermented medium, living cell mass, and impurities (dead cells and other precipitations). The environmental conditions created for cultivation have enabled researchers to perform long-term experiments without fear of contamination, and have therefore been key in understanding the metabolism of particular substrates, as well as discovering the role of specific enzymes in biochemical pathways (Schiraldi and De Rosa, 2002).

A continuous fermentation method known as the *permanent milking process* was developed, allowing companies such as Bitop AG to produce ectoine on a metric-ton scale (Lentzen and Schwarz, 2005, 2006). Due to its ability to increase the acquired cell mass, a two-step fed-batch fermentation process was used over batch fermentation to optimize the combined production of ectoine and poly(β -hydroxybutyrate) (Quillaguamán et al., 2008; Guzmán et al., 2009). The maximum production of ectoine, 5.7 g/L at 12.5% w/v NaCl with a volumetric productivity of 3.4 g/L per day, was observed. Many research groups have been exploring the opportunity to develop a process that decreases the required salt concentration while increasing the yield of ectoine, but few have succeeded in establishing a technique with results that are as promising as those described by Sauer and Galinski (Guzmán et al., 2009).

Bitop AG has developed a novel process for the production of compatible solutes, particularly ectoines. The process of permanent milking uses external filtration modules and cell recycling to achieve high-density cultivation of selected halophilic bacteria. This exposes the biomass to a successive hypoosmotic shock and results in the secretion of compatible solutes that can be synthesized and accumulated intracellularly during cultivation. The cells are then returned to high-salinity conditions with fresh culture medium and the process is repeated (Echigo et al., 2005).

15.4. COMMERCIALIZATION OF EXTREMOLYTES AND EXTREMOZYMES

The many biotechnological applications of extremolytes and extremozymes have transformed a variety of industrial products and processes. Since ancient times, enzymes have been used in the food industry for the production of cheese, beer, vinegar, and wine; however, historically, the enzymes used in these processes were produced spontaneously from growing microorganisms or were added in an impure manner (Kirk et al., 2002). By optimizing fermentation processes, pure and well-characterized enzymes are being made available for specific industrial processes within the detergent, textile, and starch industries (Schäfer, 2005). Additional advancements in biotechnology, including protein engineering and directed evolution, have made it possible to modify the enzymes to further expand their use in industrial and therapeutic applications (Kirk et al., 2002; Kumar et al., 2011).

Protein engineering has revolutionized enzyme production by adding the possibility of making small changes to protein structure and, inevitably, the related biochemical and biophysical properties (Kirk et al., 2002). Directed evolution takes this concept a step further by generating a molecular library containing all mutations and their effects. This library is used during a screening and selection process, where the improved variant of an

TABLE 15.4. Major U.S. Patents of Extremolytes and Extremozymes and Their Production Process

Patent Number	Patent Year	Author	Patent Major
3940492	1976	Ehnstrom, L.K.J., Tullinge, S.W.	Continuous fermentation process
5830696	1998	Short, J.M.	Directed evolution of thermophilic enzymes
5939250	1999	Short, J.M.	Production of enzymes having desired activities by mutagenesis
6267973	2001	Motitschke, L., Hansjorgen, D., Erwin, G.	Ectoine and ectoine derivatives as moisturizers in cosmetics
6280926	2001	Short, J.M.	Gene expression library produced from DNA from uncultivated microorganisms and methods for making the same
6171820	2001	Short, J.M.	Saturation mutagenesis in directed evolution
6632600	2003	Short, J.M.	Altered thermostability of enzymes
2004/0091932	2004	Nutsibidze, N.N., Moore, M.P., Brinkhaus, F., Salamone, P., Fonda, M., Brown, M.P., Loser, K., Gennity, I., Everett, N.	Method for producing from microbial sources enzymes having multiple improved characteristics
10504505	2005	Chew, L.C., Lee, S.L., Talbot, H.W.	Overexpression of extremozymes genes in pseudomonads and closely related bacteria
6855365	2005	Short, J.M., Kretz, K.A., Gray, K.A., Barton, N.R., Garrett, J.B., O'Donoghue, E., Mathur, E.J.	Recombinant bacterial phytases and uses thereof
10563587	2006	Krutmman, J.	Use of osmolytes obtained from extremophilic bacteria, the production of inhalable medicaments for the prophylaxis and treatment of pulmonary and cardiovascular diseases, and an inhalation device comprising an osmolyte as an active agent component
2007/0122464	2007	Krutmman J.	Use of osmolytes obtained from extremophilic bacteria for producing medicine for the external treatment of neurodermatitis
7291460	2007	Lafferty, M., Tweedy, C., Butler, J.	Multiplexed systems for nucleic acid sequencing

organism is then used as the starting material for a new recombination in the next generation (Kirk et al., 2002). Many properties of extremolytes and extremozymes and their processes of manufacture have been patented, some of which are summarized in Table 15.4.

Verenium Corporation has brought to market nine industrial enzymes using the patented Direct Evolution technology (Table 15.4). The patent claims a method for developing thermostable enzymes via mutagenesis of thermophiles. These enzymes are not only active at high temperatures ($>60^{\circ}\text{C}$), but also twice as active as the enzymes produced from

the wild-type strain at relatively low temperatures ($<50^{\circ}\text{C}$). In 2009, production of these enzymes resulted in a revenue of \$49 million for the company (www.verenium.com). To achieve such success, Verenium extracts DNA directly from microbes isolated from various environmental samples, ranging from volcanoes to the tundra, the tropics, and the desert. This method avoids the culturing process and has enabled the generation of large and complex microbial gene libraries (www.verenium.com). Gene clusters are structures located on a single chromosome, whose products aid in identical or related functions. Each individual gene in the cluster is transcribed together after a promoter sequence initiates the transcription process. A combination of the promoter, gene cluster, and any additional sequences that regulate the resulting protein expression is known as an *operon*. Operons can contain up to 20 or more genes and undergo continual reorganization. The generation of gene cluster libraries has enabled researchers to identify the nucleotide sequences that result in desirable protein expressions.

The gene cluster that results in the enzymes responsible for polyketide synthesis has been identified and cloned successfully. Polyketide synthases are multifunctional enzymes that catalyze the synthesis of a wide range of biological molecules, including antibiotics (such as tetracycline and erythromycin), anticancer agents (daunomycin), immunosuppressants (FK506 and rapamycin), and veterinary products (monensin). To clone gene clusters, the operon is ligated into a fertility factor (or f-factor)-based vector, such as *E. coli*, which regulates production of the proteins specified. The vector is capable of transferring its entire DNA sequence at a high frequency during conjugation, making it ideal for quick and stable propagation of the operon (Table 15.4).

Verenium's proprietary technology, known as the GigaMatrix ultrahigh-throughput screening platform (U.S. Patent 7291460, Table 15.4) is designed to mine genetic libraries for novel biomolecules and is capable of screening the nucleic acid sequences of 1 billion clones per day. Each plate that is screened contains up to 1 million wells, which require a minuscule sample volume, increasing the speed of the screening process and making it more cost-effective. The resulting Verenium enzyme products fall into one of four categories: animal health and nutrition, oilseed processing, grain processing, or emerging enzyme markets.

Bitop AG uses a continuous fermentation method to produce Ectoin on a large scale for a variety of applications, ranging from cosmetics to therapeutics. However, recent research has led to the identification of the complete genome sequence of *H. elongata*, as well as the biosynthetic pathway for ectoine production and degradation (Schwibbert et al., 2011). The three genes studied (*ectA*, *ectB*, and *ectC* cluster) were found to be involved in ectoine biosynthesis (Schubert et al., 2007; Schwibbert et al., 2011). The cells take up ectoine or precursors for it from the medium using an ectoine-specific membrane transporter known as TeaABC (Grammann et al., 2002). Genencor has commercialized extremozymes for use in textile detergents, including several other examples reviewed by Antranikian et al. (2005).

15.5. PRODUCT RECOVERY

After a successful fermentation, recovery of the purified product is equally important. Zhang et al. (2009) separated the cells from the fermentation broth using centrifugation at $16,000 \times g$ for 15 min, and the supernatant was analyzed using high-performance liquid

chromatography (HPLC). They also used a 0.2- μ m-pore-size membrane filter to separate the cells at the exponential phase of growth. Intracellular ectoine measurement was achieved by washing the pellets with 100 mM/L KP_i buffer (pH 7.2) containing NaCl and extracting the compatible solutes using 80% ethanol. The suspension was centrifuged again and the supernatant was analyzed using HPLC at 210 nm. Extracellular ectoine measurement was obtained by centrifuging or filtering the suspension, and the supernatant was diluted 10-fold with distilled water prior to HPLC analysis (Zhang et al., 2009).

Guzmán et al. (2009) extracted the compatible solutes by exposing them to hypoosmotic shock. The concentrations of compatible solutes and MSG were measured using HPLC at 210 nm, and the chemical structures were identified using proton nuclear magnetic resonance (1H -NMR) analysis (Guzmán et al., 2009).

15.6. CONCLUSIONS

Industrial processes have created a demand for extremozymes. Extremophiles have strong implications for future advancements in biotechnology, pharmaceuticals, and the extermination of certain toxic compounds. Because of extremophiles' strong flexibility in surviving under extreme habitats that would normally kill an organism almost instantly, extremozymes such as thermostable amylases are being incorporated into biochemical reactions that occur at very high temperatures in water-based solutions. Other high-cost reactants can be replaced with extremozymes to lower the cost of their products. In addition, radiation-resistant microbes contain compounds that can potentially be harnessed as radioprotective drugs, which have enormous potential for use in space programs and to prevent unwanted radiation exposure. Because of the versatility of these microorganisms, further studies on microbial ecology and taxonomy will investigate extremophiles and their metabolic products of commercial interest. In the years to come, further exploitation of these extremophiles will indubitably advance toward finding cures for diseases such as radiation-mediated cancer and in meeting other industrial demands.

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SMART THERAPEUTICS FROM EXTREMOPHILES: UNEXPLORED APPLICATIONS AND TECHNOLOGICAL CHALLENGES

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16.1. INTRODUCTION

The term *extremophiles* is used to categorize a specific group of organisms, including plants, animals, and microorganisms that thrive under extreme physical, chemical, and nutritional environmental conditions, such as hot springs, volcanic high-temperature and high-pressure zones, the deep-sea high-pressure environment, extremely high altitude with ultralow temperature and pressure, extremely high and low temperature ($>45^{\circ}\text{F}$; $<15^{\circ}\text{C}$), pressure, oxygen scarcity (oxidative stress), and a variety of electromagnetic radiations, including nonionizing [ultraviolet (UV)] as well as ionizing (gamma) radiation, infrared thermal radiation, and high-frequency microwave (Mesbah and Wiegel, 2008; Kumar et al., 2010). The term *extremophile* was introduced by MacElroy in 1974, although the specific terms *acidophile*, *halophile*, and *thermophile* have been used in the literature since about the turn of the twentieth century. Extremophiles can be grouped according to the conditions in which they thrive. Thus, they can be categorized into the following types on the basis of their habitats: thermophiles, psychrophiles, barophiles or piezophiles, halophiles, alkaliphiles, and acidophiles (Table 16.1).

Extremophiles: Sustainable Resources and Biotechnological Implications, First Edition. Edited by Om V. Singh.
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TABLE 16.1. Typical Extremophile Genera Thrive Under Extremely Classified Conditions

Type	Growth Characteristics	Typical Genera
Hyperthermophiles	>80°C	<i>Aquifex</i> , <i>Thermococcus</i> , <i>Thermoproteus</i> , <i>Pyrolobus</i> , <i>Sulfolobus</i> , <i>Hydrogenetobacter</i> , <i>Methanothermus</i> , <i>Pyrococcus</i>
Thermophiles	60–80°C	<i>Methanobacterium</i> , <i>Thermus</i> , <i>Thermoplasma</i> , some <i>Bacillus</i> spp.
Psychrophiles	<15°C	<i>Psychrobacter</i> , <i>Alteromonas</i>
Halophiles	High salt (e.g., 2–5 mM NaCl)	<i>Halorubrum</i> , <i>Haloferax</i> , <i>Halobacterium</i> , <i>Haloarcula</i>
Alkaliphiles	pH > 9	<i>Natronococcus</i> , <i>Natronobacterium</i> , some <i>Bacillus</i> spp.
Acidophiles	pH < 4	<i>Thiobacillus</i> , <i>Sulpholobus</i> , <i>Desulfurolobus</i> , <i>Acidianus</i>

Extreme environments disturb the homeostasis of the human body and thus could lead to deleterious disturbances in the normal functioning of the body. General medical problems encountered in extreme environments include sunburns due to high UV radiation flux, hypertension due to low pressure, cold shock and frostbite due to ultralow temperatures, heat shock in high-temperature areas, disturbances in membrane permeability, ion misbalances, decrease in metabolic rate leading to laziness, oxidative stress due to lack of oxygen, and high radiation background leading to DNA damage and cell death. Extremophiles can survive under extreme environments, carrying novel in-built machinery that protects life in harsh environments. This may open up a vast panorama of extremophile exploitation for their potential therapeutic use to counter diseases and disorders induced as a result of harsh environments.

Experimental studies indicate that extremophiles have adopted a variety of ingenious strategies for survival under conditions of high or low temperature, extreme pressure, and drastic salt concentrations by synthesizing *extremolytes* (i.e., organic osmolytes) to protect biological macromolecules and cells from damage by external stresses (Lentzen and Schwarz, 2006). These low-molecular-mass compounds have accumulated in response to extreme environmental changes and minimize the denaturation of biopolymers. For example, the ectoines, the first extremolytes being produced on a large scale, have proved their value as cell protectants in skin care products and as protein-free stabilizers of proteins in other biological products. In addition to ectoines, a range of extremolytes with heterogeneous chemical structures, such as the polyol phosphates dimyoinositol-1,1'-phosphate, cyclic 2,3-diphosphoglycerate, and α -diglycerol phosphate, and the mannose derivatives mannosylglycerate (firoin) and mannosylglyceramide (firoin A) (Lentzen and Schwarz, 2006) were characterized for their protein-protecting potential against oxidative and related stress.

A range of new applications, all based on adaptation to stress conditions conferred by extremolytes, are already in the developmental stage (Lentzen and Schwarz, 2006). γ -Diglycerol phosphate (DGP) was identified as a new therapeutic agent extracted from *Archaeoglobus fulgidus* and shown to be an effective protein stabilizer in vitro (Lamosa

et al., 2003; Pais et al., 2005). DGP is also known to accumulate in response to elevated salinity (Lamosa et al., 2000). Archaeobacteria are well known as extremophilic candidates among the microorganisms. Typical halotolerant and hyperthermophilic archaeobacteria *Pyrococcus furiosus* and *Thermotoga maritima* accumulate negatively charged derivatives of inositol and glycerol at extreme temperature and high salt concentrations (Scholz et al., 1992; Ciulla et al., 1997). In this chapter we provide extremophile-mediated therapeutic implications of products of human concern obtained from extremophiles (extremolytes). We also highlight the existing and futuristic potential of extremophiles in therapeutics due to unusual medical issues. Our discussion explores certain ways to fill the technological gaps and overcome the existing challenges to achieve set goals in extremophilic biotechnology.

16.2. EXTREMOLYTES AS PROTEIN PROTECTANTS

Functional proteins are the ultimate downstream regulators of various metabolic, cellular, and molecular reactions. Folding and misfolding of proteins are among deciding factors toward targeted functions of specific protein. Various stresses responsive to protein function alter their folding and/or misfolding process. Arakawa and Timasheff (1985) proposed that protein-protecting extremolytes have increased (preferential) hydration of the protein, which favors the native state of the protein without interacting directly with the surface of the proteins. Because osmolytes produced by extremophiles do not interact directly with proteins, the catalytic activities of enzymes are largely undisturbed.

Extremolytes such as ectoines play an important role in the inhibition of amyloid formation (Lentzen and Schwarz, 2006). The amyloid is a protein aggregation leading to the formation of highly regular aggregates in the number of diseases associated with the misfolding of proteins, such as Alzheimer's disease and spongiform encephalopathies (Dobson, 2003). In a study that used the formation of insulin amyloid in vitro as a model system, it was shown that ectoine is a very effective inhibitor of amyloid formation, decreasing both the initiation and elongation phases of amyloid formation (Arora et al., 2004). Subsequent study unravels the influence of ectoine and hydroxyectoine on A β peptide amyloid formation (Kanapathipillai et al., 2005). A β peptide is the major constituent of senile plaques, the key pathological feature of Alzheimer's disease. Ectoine and hydroxyectoine were both effective inhibitors of A β 42 aggregation and associated toxicity in human neuroblastoma cells. Protein misfolding is also a key event in the pathogenesis of polyglutamine diseases such as Machado–Joseph disease (MJD). Truncated MJD gene product with an expanded polyglutamine tract induced apoptotic cell death in cultured neuro-2 α neuroblastoma cells. Ectoine has been reported to decrease apoptosis in Machado–Joseph disease by reducing protein aggregates, resulting in the inhibition of structural changes leading to intracellular distribution (Furusho et al., 2005).

16.3. EXTREMOLYTES AS CELL PROTECTANTS

Extremolytes are able to stabilize proteins and other macromolecules; they also play a significant role in cell protection against oxidative stress. The protective effect of extremolyte produced by extremophiles is well known in both prokaryotes and higher eukaryotes, including animals and human cells. UV subtype A (UVA)–irradiated human keratinocyte

cells were found to be protected from damage when pretreated with ectoine (Bünger and Driller, 2004). The same study further revealed that the UVA-induced second messenger release, transcription factor AP-2 activation, intercellular adhesion molecule-1 expression and mitochondrial DNA mutation can be prevented by ectoine pretreatment to human keratinocyte cells (Bünger and Driller, 2004). This shows an ectoine-mediated mechanism that could stabilize the membrane structures, leading to increased resistance to deleterious damage induced by UVA. Ectoine has also been studied in reducing or protecting cells against UVA-induced sunburn (Bünger et al., 2001). Ectoine-mediated neutralization of free radicals induced by UV radiation (UVR) has been reported to be useful in preventing water loss in dry atopic skin and can prevent skin aging. Ectoine pretreatment to Langerhans cells showed significant protection against UV radiation toward Langerhans cell-mediated T-cell immune response (Beyer et al., 2000) and thereby exerts an immunoprotective effect along with skin protection from potential damage from UV radiation. Buommino et al., (2005) showed the cytoprotective effect of ectoine against sepsis caused by bacterial lipopolysaccharide, by inducing elevated levels of Hsp70 protein. Extremolytes provide cell protection against a drying environment, probably by replacing water molecules with the hydroxyl group of ectoine (Lippert and Galinski, 1992) and thus stabilize membrane fluidity. Hydroxyectoine (4S-2-methyl-1,4,5,6-tetrahydropyrimidine-4-carboxylic acid) was discovered originally from an extremely halophilic phototrophic eubacterium *Halorhodospira halochloris*, isolated from Wadi Natrun in Egypt (Galinski et al., 1985). In addition to osmolytes, other compounds were also isolated from extremophiles and reported to have therapeutic potentials. Some of these are described below.

16.3.1. Mycosporine-like Amino Acids

The mycosporine-like amino acids (MAAs) are known to absorb UVR in the wavelength range 310 to 365 nm (Singh and Gabani, 2011) and are found in a wide range of microorganisms, including cyanobacteria and eukaryotic algae. UVR forms pyrimidine dimers in the DNA, which ultimately results in mutations at the site of pyrimidine dimer formation; if these types of mutations occur in a cell cycle regulatory gene (i.e., p53), normal cells may lose control on cell cycle, possibly resulting in normal cells transformed to cancerous cells. MAAs are known to protect against UVR-induced DNA damage by preventing the formation of DNA dimers. Oyamada et al., (2008) also reported protective effects of MAAs against UV radiation on the growth of human skin fibroblast cells. Because MAAs are regarded as UV radiation absorbers, currently they are being used in UV sunscreens in the cosmetics industry and have been suggested for potential applications in the prevention of cancer induced by UVR, such as melanoma (de la Coba et al., 2009). Other reports demonstrated that a formulation containing MAA (prophyra-334 and shinorine, i.e., P-334 + SH) was found preventive against sunburn, in corneum stratum, malpighian, dermal and hypodermal thickening, and other structural and morphological alterations (Conde et al., 2000). The formulation of MAA showed protection against UV-induced skin damage in mice, and its effect was also reported to be associated with maintaining the antioxidant defense system of the skin (de la Coba et al., 2009). Various other MAAs that have been suggested for photoprotection include palythine, asterina, palythinol, and palythine. Palythine has been found in many species of microalgae, including *G. galatheanum* and *G. venificum* (Llewellyn and Airs, 2010). Maristentorin is found in *Heterotrich ciliate* and *Maristerntor dinoferus*. A role for maristentorin similar to that for stentorian and blepharismim suggests

potentially similar biological functions (i.e., defense against UV irradiation) (Mukherjee et al., 2006). Although these compounds are promising, direct therapeutic implications of MAAs as drug candidates in humans are still awaited (Conde et al., 2003).

16.3.2. Bacterioruberin

The microorganism *Rubrobacter radiotolerans* is extremely resistant to the lethal effects of ionizing radiation. The resistance of this bacterium to ionizing radiation was found to be greater than that of the well-known radioresistant bacterium *D. radiodurans* (Asgarnai et al., 2000). It has been shown that *Halobacterium salinarium*, another red-pigmented bacterium containing bacterioruberin, is extremely resistant against ionizing radiation (Asgarnai et al., 2000). Shahmohammadi et al., (1998) reported that this organism is highly resistant to the lethal action of DNA-damaging agents such as gamma and UV radiation, indicating a direct correlation between the presence of bacterioruberin and repair mechanisms involved in DNA repair. Therefore, the DNA repair role of bacterioruberin in radioresistant microbes could be utilized in humans to repair damaged DNA strands caused by ionizing radiation.

16.3.3. Sphaerophorin and Pannarin

Among UV-tolerant organisms, the lichens are symbiotic organisms of fungi and algae/fungi, and cyanobacteria producing secondary metabolites of pharmaceutical significance (Muller, 2001). Secondary metabolites obtained from lichens have been considered for use in sunscreen for UV protection because of their antioxidant activity. Owing to their geographical location, Chilean lichens are able to exhibit protection against intense UVR. The effect of two lichen compounds, sphaerophorin and pannarin, was evaluated on pBR322 DNA cleavage induced by hydroxyl radicals (OH^-) and nitric oxide (NO^-), and also for their superoxide anion (O_2^-) scavenging capacity (Russo et al., 2008). Cell culture experiments showed that these compounds inhibited the growth of human melanoma cells (M14 cell line) by inducing apoptosis (Russo et al., 2008) and thus could be used as anticancer agents. These studies support further research on the molecular mechanisms underlying sphaerophorin and pannarin biological activity in in vitro and in vivo models.

16.4. NOVEL THERAPEUTICS IN THE DEVELOPMENTAL STAGE

Various nonconventional sources, such as deep-sea macro- and microflora have been studied to isolate novel therapeutic agents that would inhibit or activate the vital signaling pathways that lead to inhibition or prevention of specific diseases or disorders. For example, a novel protein kinase inhibitor, bryostatin, isolated and purified from the bryozoan *Bugula neritina*, is being evaluated for its anticancer properties. Several other marine microalgae, heterotrophic bacteria, and cyanobacteria living in symbiotic association with invertebrates (e.g., sponges, tunicates, soft corals) have also been identified as the original sources of many therapeutically active compounds, such as kahalalide F, E7389, curacin A, salinosporamide A, and eleutherobin, and may be important drug candidates for use in humans (Bai et al., 1991; Hamann et al., 1996; Haefner, 2003; Simmons et al., 2005). Similarly, a novel protein serine/threonine kinase inhibitor, scytonemin, isolated from an

extremophilic marine cyanobacterium, *Stigonema* sp., collected from Waldo Lake, Oregon, has tremendous therapeutic applications (Stevenson et al., 2002).

Other important biomolecules and secondary metabolites of therapeutic significance have been screened to cure cancer and related diseases. A battery of such compounds of therapeutic importance has been explored in the extremophilic marine organisms (Edler et al., 2002; Gamble et al., 1999; Hood et al., 2001; Lifeng et al., 2001; Luesch et al., 2002; Mooberry et al., 1999; Poncet, 1999). Two important carbohydrate extremolytes, mannosylglycerate (firoin) and mannosylglyceramide (firoin A), accumulate in the cells in response to heat stress in the thermotolerant bacterium *Rhodothermus marinus*. Both mannosylglycerate and mannosylglyceramide accumulate in host cells in response to thermal stress, while uncharged mannosylglyceramide increased in cells with elevated NaCl levels (Karsten et al., 1993). Di-myo-inositol-1,1'-phosphate (DIP), a phosphodiester derivative of myoinositol, provides protection against salinity. γ -Diglycerol phosphate (DGP) was identified as a new extremolyte in *Archaeoglobus fulgidus* and shown to be an effective protein stabilizer in vitro (Lamosa et al., 2003; Pais, 2005). DGP is also known to accumulate in response to elevated external NaCl concentrations, while temperature increases lead to enhanced DIP accumulation (Lamosa et al., 2000). Similarly, cyclic 2,3-diphosphoglycerate (cDPG) and cyclic trianionic pyrophosphate were found to accumulate in the archaea *Methanothermobacter thermoautotrophicus*. The primary role of cDPG in *Methanothermobacter* may be as a phosphate storage compound, which may provide protection to glyceraldehyde-3-phosphate dehydrogenases at high temperature (Hensel and Jakob, 1994; Matussek et al., 1998). Various other extremophiles and their extremolytes are summarized in Table 16.2.

16.5. HOMELAND SECURITY AND MILITARY MEDICINE

Exploration of strategic therapeutic applications of extremophiles in the area of defense and homeland security has credible potential. Although much of the literature is not available, scientists are exploring the hidden potential of extremophiles for strategic applications. The development of radioprotective drugs using radioresistant bacteria is an important task undertaken by INMAS in Delhi, India. The radioresistant bacterium *Bacillus* sp. INM-1 was isolated and characterized. The antioxidant, immunomodulatory, and radioprotective efficacy of bimolecular entities extracted and purified from fermentation broth of the radioresistant bacterium *Bacillus* sp. INM-1 was evaluated (Kumar, 2011a,b). Similarly, the in vitro radioprotective efficacy of the Mn^{+} decapeptide complex isolated from the world's most radioresistant bacterium, *Deinococcus radiodurans* R1, has been evaluated (Daly et al., 2010). The development of radioprotective drugs is a dream of radiobiologists worldwide. Therefore, radioresistant microorganisms may be the potential source of efficacious and nontoxic radioprotective drugs in the future.

Another important area of extremophile application is in the medical management of blast- and shock wave-induced traumatic brain injuries in soldiers operating in low-intensity war zones. Blast waves generated by explosive detonation generate a powerful shock wave with high-pressure intensity. Interaction of high-pressure shock waves with brain tissues induced undetectable injuries which may lead to various types of disorders, such as sleeplessness, short- and long-term memory loss, anxiety, stress, fatigue, and depression leading to neurocognition impairment (Cernak and Haeusslein, 2010; Risdall and Menon, 2011). Therefore, to address shock wave-induced biological damage and its

TABLE 16.2. Sustainable Resources of Novel Extremolytes and Their Applications

Source of Novel Therapeutic Agents	Novel Therapeutic Agents	Therapeutic Applications	References
<i>Halorhodospira halochloris</i>	Ectoine	Stabilization of proteins and enzyme against thermal, cold, and desiccation Protection of lactate dehydrogenase against heat and freeze–thawing cycling Inhibitory effect on insulin amyloid formation Stabilization of tobacco cells against hyperosmotic stress Block of UVA-induced ceramide release in human keratinocytes and inhibition of apoptosis Reduction of the water loss and dryness from skin Radioprotection of skin immune cells Cryoprotection of keratinocytes Protein protection against oxidative damage Reduction in immunotoxine virulence Retroviral vaccine stabilization Thermotolerance induction Anticancer Protein thermostabilization Enzyme stabilization against cold and heat stress	Lippert and Galinski, 1992 Göller and Galinski, 1999 Arora et al., 2004 Nakayama et al., 2000 Grether-Beck et al., 2005 Bünger and Driller, 2004 Beyer et al., 2000 Buommino et al., 2005 Andersson et al., 2000 Barth., 2000 Cruz, et al., 2006 Malin and Lapidot, 1996 Simmons et al., 2005 Lamosa 2000,2003 Borges et al., 2002; Ramos et al., 1997 Bai et al., 1991 Hamann et al., 1996
<i>Streptomyces</i> strain	Hydroxy ectoine		
<i>Lyngbya majuscula</i> <i>Archaeoglobus fulgidus</i> <i>Rhodothermus marinus</i>	Curacin A Diglycerol phosphate Mannosylglycerate		
<i>Halcondria okadae</i> <i>Elysia rufescens</i> / <i>Bryopsis</i> sp.	E7389 Kahalalide F	Anticancer Antipsoriasis	

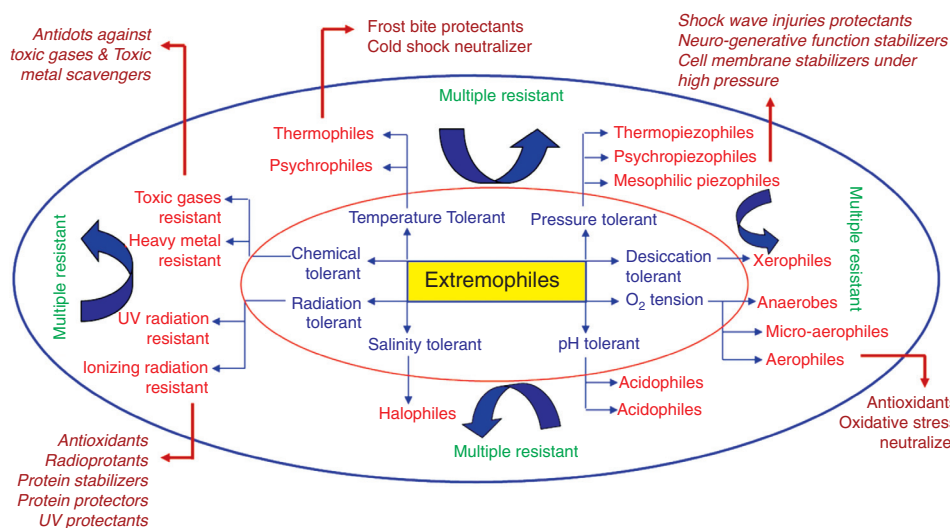


Figure 16.1. Major groups of extremophiles (inner circle) and their interrelationships lead to multiple resistance (outer circle) and possible therapeutic implications. (See *insert* for color representation of the figure.)

repair, deep-sea inhabitants, piezophilic microbes surviving extremely high-pressure zones in the deep sea, could be explored as the credible sources of novel drugs to modulate high-pressure blast and shock wave injuries. Most piezophilic microbes are known to sustain their lives by maintaining cell membrane integrity even at extremely high atmospheric pressure in the deep marine subsurface. This particular feature of piezophilic microbes can be explored to develop antipressure medicine for military and civilian use. Although the potential seems to be there, due to a lack of consolidated effort, limited progress has been made in exploring the applications of piezophilic microbes in blast- and shock wave-induced brain injuries (Fig. 16.1).

16.6. TECHNOLOGICAL GAPS IN THERAPEUTIC PRODUCT DEVELOPMENT USING EXTREMOPHILES

Even after recognition of undisputed therapeutics and industrial applications of extremophiles in biotechnology, overall progress is sluggish. The prime reasons could be due to technological gaps which still persist and are a major obstacle to transforming the theoretical concepts into real practical approaches (Fig. 16.2).

Several prominent technological gaps have been identified:

1. Real simulated extreme environments such as high background UV/ionizing gamma radiation, high-pressure, shock wave-simulated, desert, and hypoxic environments need to be created in experimental setups (e.g., an innovative fermentation process) to mimic real situations prerequisite for appropriate response of a particular extremophilic organism in the extreme environment simulated.

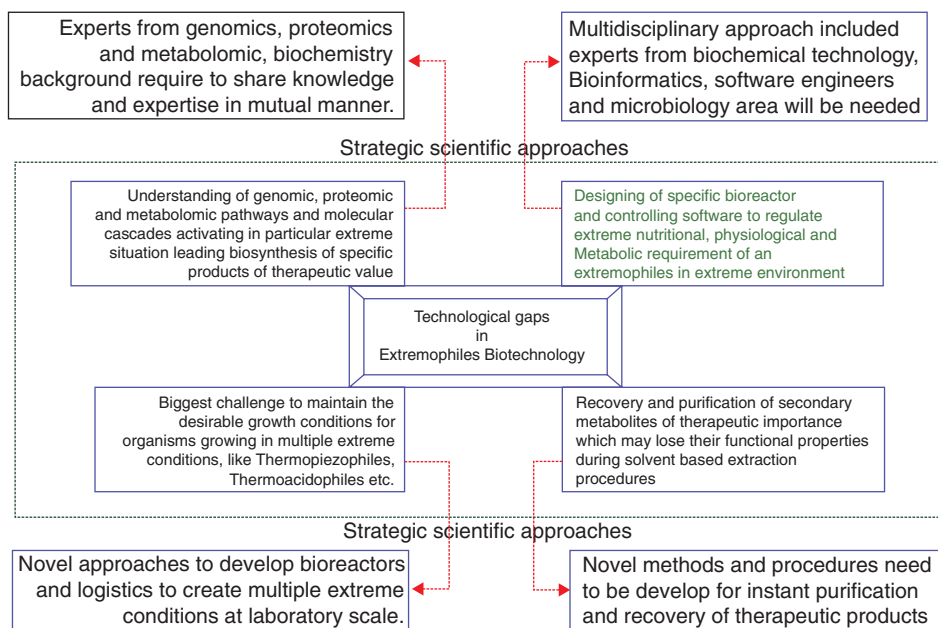


Figure 16.2. Prominent technological gaps and possible outcomes to explore extremophiles and novel therapeutically important product development.

2. Complete genomic, proteomic, and metabolomic pathways and molecular cascades activated in particular extreme situations need to be understood prior to exploring desired applications.
3. Specific dedicated bioreactor systems able to create an extreme environment inside a culture chamber need to be developed.
4. Specific nutritional requirements of extremophiles to synthesize specific metabolites of therapeutic applications in controlled culture conditions need to be unveiled.
5. Rapid extraction, purification, recovery, and preservation of a desired therapeutic product synthesized by an extremophilic organism need to be discovered. Most secondary metabolites may lose their functional properties because they are simply not stable in organic solvents and under normal ambient working environmental conditions. Therefore, it is most important to determine the most suitable extraction, purification, and storage conditions for perishable extremophilic products of therapeutic significance.

16.7. CONCLUSIONS

The aim of this chapter was to highlight the existing and futuristic potential of extremophiles in therapeutics for unusual medical issues. Because of the complex physiology of extremophilic organisms, advanced approaches in modern biology, such as genomics, proteomics, and metabolomics, may provide additional information regarding the response of

physiological changes under a variety of conditions. Investigation of extremolytes from extremophiles has progressed slowly, due to persistent technological gaps; however, the rate of development depends largely on economic advantages for biopharmaceutical industries and their interest in novel and economically viable molecules from extremophiles. Even though advances have been made in recent years, current knowledge is still relatively limited. Increased research effort will assist in investigating the unique properties of extremophiles, which clearly have viability in therapeutic applications for military personnel as well as for civilians.

Acknowledgment

Thanks to Om V. Singh for his valuable suggestions and for giving us the opportunity to share our views on therapeutic implications of extremophiles. His efforts in promoting extremophile research are deeply appreciated.

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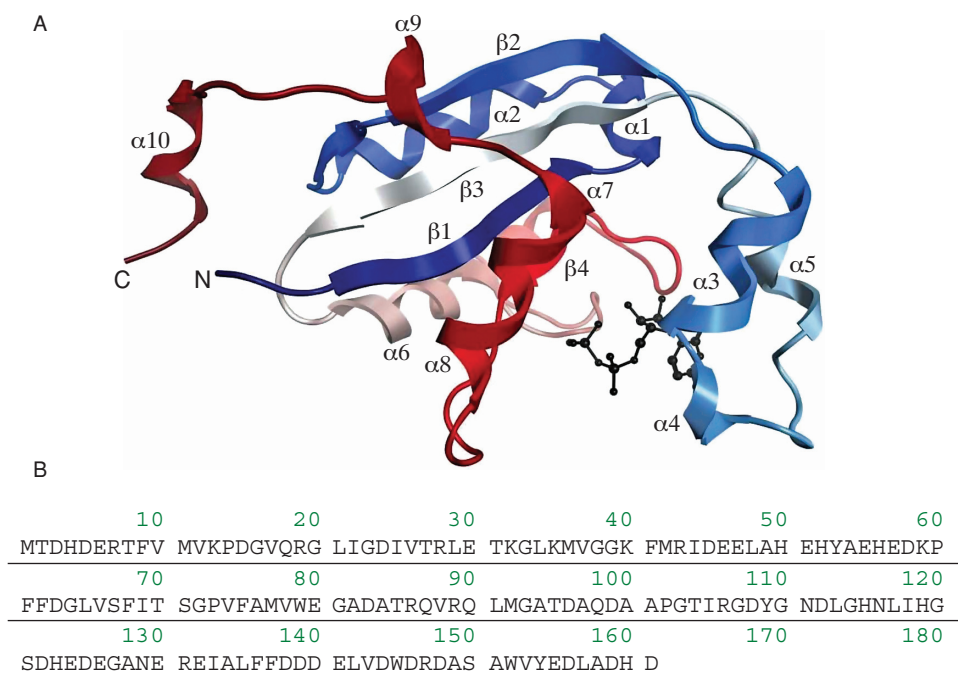


Figure 4.3. Structure (A) and sequence of HsNDK (B). The protein backbones of the monomer subunit are shown as blue to red from the N-terminus to the C-terminus (A). Chain termini and secondary structure elements were indicated (B). CDP was shown in a ball-and-stick representation.

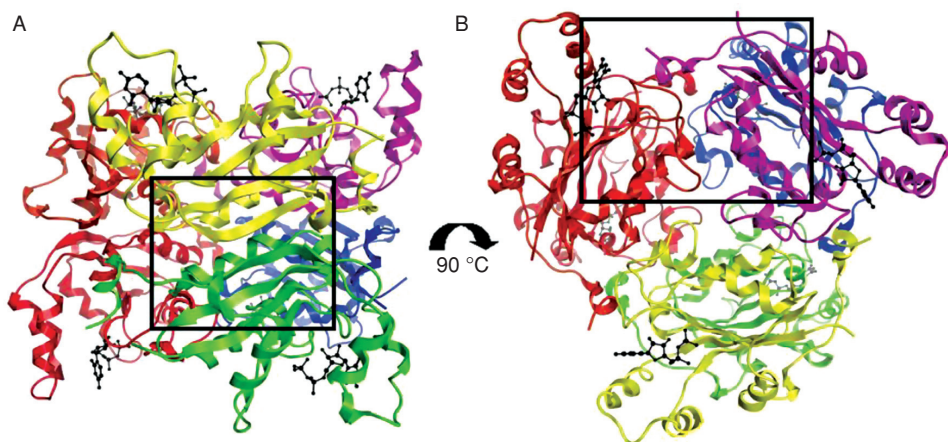


Figure 4.4. Hexameric structure of HsNDK; each monomer is depicted in different colors. Dual colors (e.g., red–brown, blue–purple) show basic dimeric units forming a hexamer. (A) Monomer–monomer interface (see the box); (B) dimer–dimer interface (see the box).

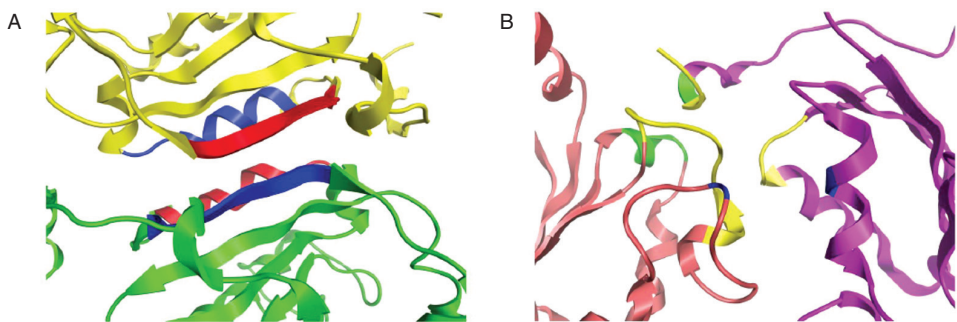


Figure 4.5. Detailed structure of monomer–monomer interface (A) and dimer–dimer interface (B). Contacts between two monomers (A) and between two dimers (B) are shown in color as described in the text. (A) Box in Figure 4.4A is expanded; (B) box in Figure 4.4B is expanded.

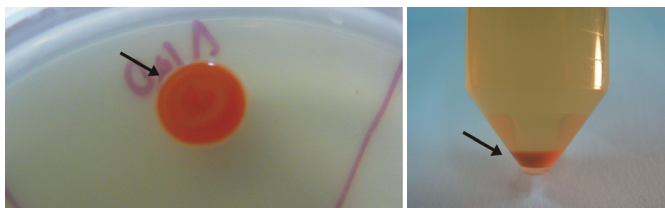


Figure 5.1. Pigmented cells in *Haloferax volcanii*. Left: spot on agar plate (arrow). Right: cells precipitated at the bottom of a liquid culture (arrow). (Courtesy of Dr. Ximena Abrevaya.)



Figure 5.2. Salt lake in Namibia, Africa. The reddish colored water is due to the presence of halophilic-pigmented microorganisms, mostly haloarchaea. (Courtesy of Dr. Karsten Kotte, Institute of Earth Sciences, University of Heidelberg.)

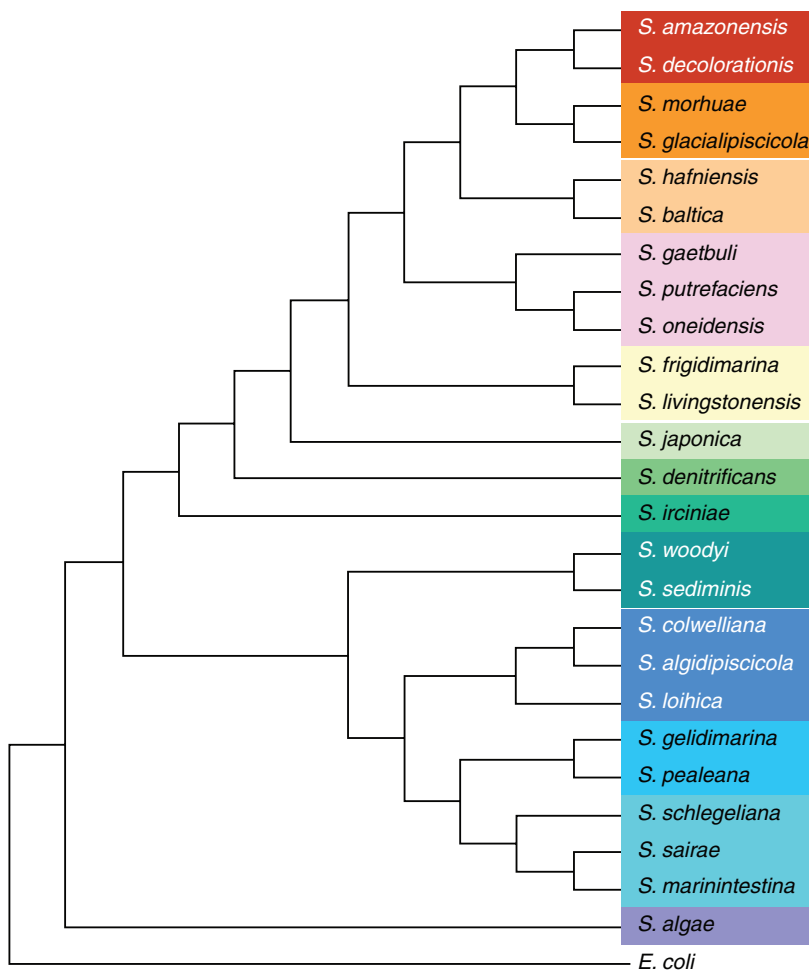


Figure 6.4. Phylogenetic tree of *Shewanella* 16 rRNA gene sequence from strains isolated from cold-temperature environments. Phylogenetic analysis was carried out by the neighbor-joining method using MEGA 5 program.



Figure 7.1. Power plant in Iceland.



Figure 7.2. Some representative hot environments: (A) fumarole at Campi Flegrei near Napoli, Italy; (B) terrestrial hot springs at Viterbo, Italy; (C) deep-sea hydrothermal vents at Okinawa Trough in Japan. (Courtesy of F. Canganella.)

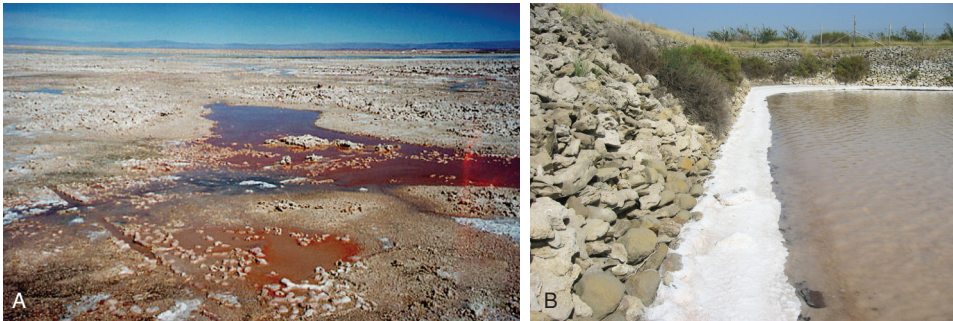


Figure 7.6. Two examples of ecosystems characterized by elevated NaCl concentrations: (A) Atacama desert (Chile); (B) Mediterranean solar salterns (Italy). (Courtesy of F. Canganella.)



Figure 7.7. Deep-sea hydrothermal vent chimney. (Modified from *Focus* magazine.)



Figure 11.2. Raw silk and degummed silk.



Figure 11.4. Enzyme-faded jeans.

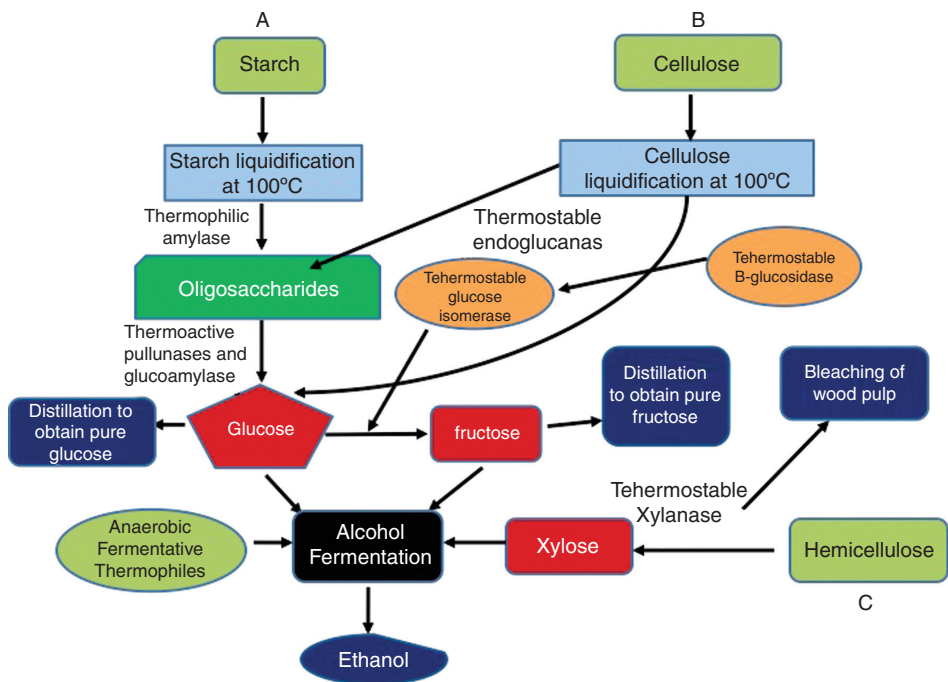


Figure 15.1. Extremozymes mediated production of sugars and ethanol from unconventional carbon sources found in thermophiles. (A) Conversion of starch; (B) conversion of cellulose; (C) conversion of hemicellulose.

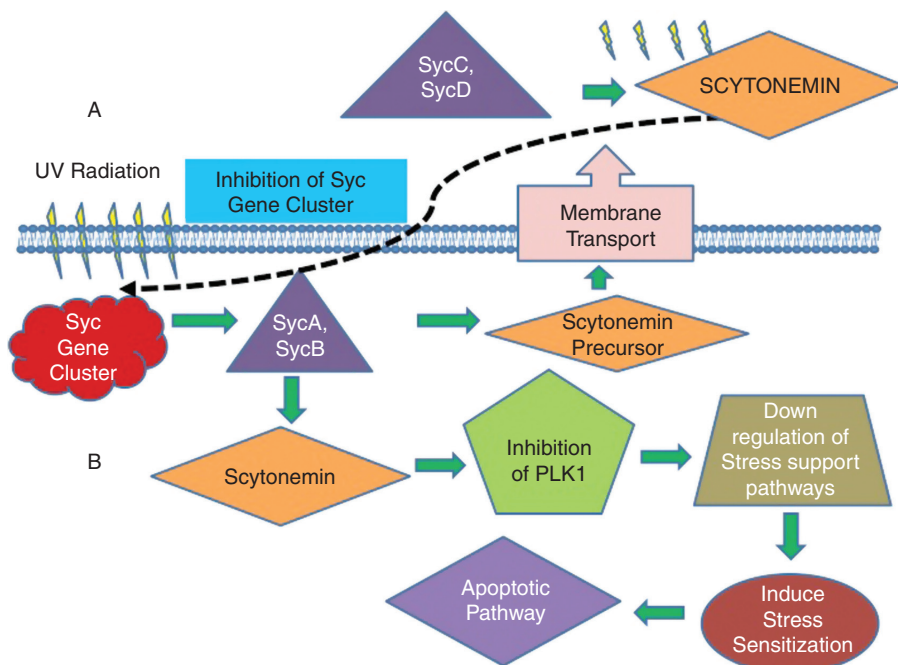


Figure 15.2. Escape mechanism from ultraviolet (UV) radiation led to therapeutics. (A) UVA is absorbed and induces the Syc gene cluster to produce scytonemin. Scytonemin is transported outside the cell membrane, where its accumulation ultimately blocks UV radiation. (B) The inhibition of PLK1 is hypothesized to induce stress sensitization by downregulating the activity of stress support pathways, leading ultimately to apoptosis of cancer cells.

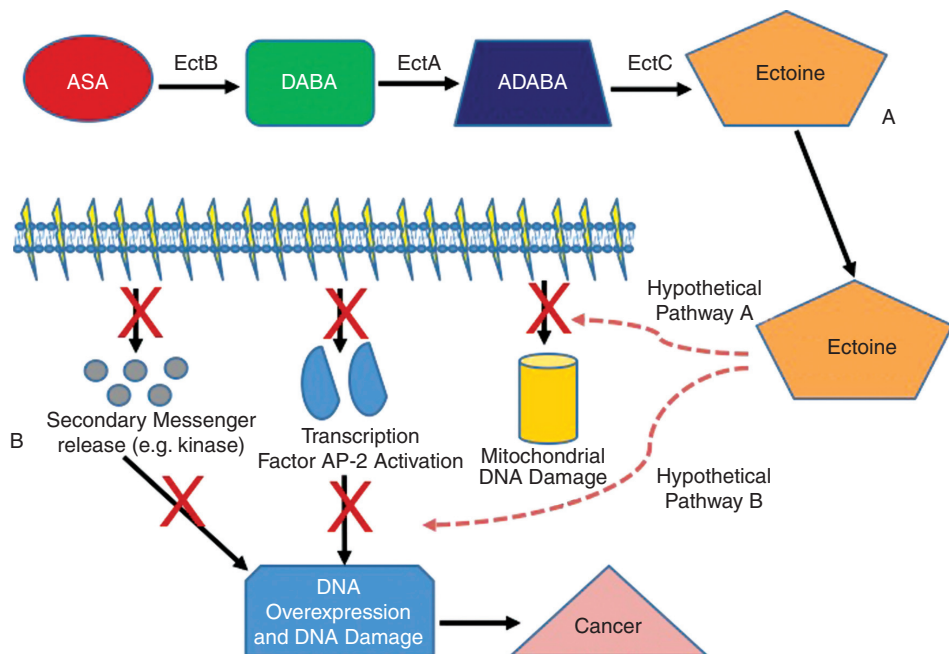


Figure 15.3. Biosynthesis of extremolytes in halophilic bacterium *H. elongate* and proposed hypothetical survival mechanism. (A) Biosynthesis of multiple ectoines in *H. elongate* (ASA, aspartate semialdehyde, DABA; 1-2,4-diaminobutyrate; ADABA, *N*-acetyl-1-2,4-diaminobutyrate). (B) Hypothetical pathway A: ectoine could act as a UV blocker and prevents the induction of secondary messengers, transcription factor AP-2 activation, and mitochondrial DNA mutations. Hypothetical pathway B: ectoine may also act as an inhibitor and blocks the secondary messengers and transcription factor AP-2 from binding to promoters and halts DNA transcription.

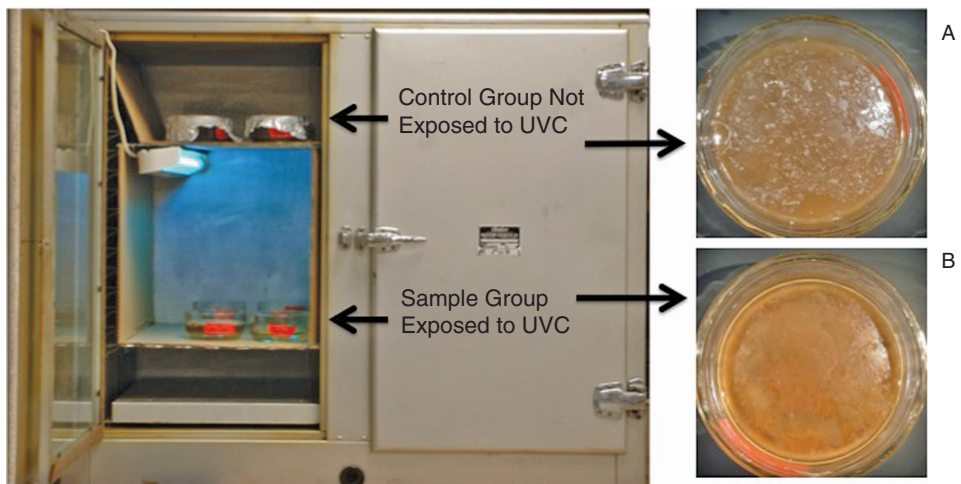


Figure 15.4. UV radiation chamber for isolation of UVR-resistant microorganisms. (A) Control group, not exposed to the UV, shows the cloudy appearance of microbial flora in nutrient broth medium. (B) Sample group, exposed to the UV, shows comparably fewer microorganisms on the surface of the medium after continuous UV exposure for 7 days in both groups at 37°C.

